SEX STEROIDS IN SJÖGREN'S SYNDROME

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of
the University of Helsinki, for public examination in Lecture hall 2 at Meilahti hospital,
Haartmaninkatu 4, Helsinki, on 25 March 2011, at 12 noon.

Helsinki 2011

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Tieteellinen tutkimus ORTONin julkaisusarja, A:31
Publications of the ORTON Research Institute, A:31

ISBN 978-952-9657-57-5 (paperback)
ISBN 978-952-9657-58-2 (PDF)
ISSN 1455-1330
http://ethesis.helsinki.fi

Helsinki University Printing House
Helsinki 2011

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which in text are referred to by Roman numerals (I-V):

I **Porola P**, Virkki L, Przybyla BD, Laine M, Patterson TA, Pihakari A, Konttinen YT. Androgen deficiency and defective intracrine processing of DHEA in the salivary glands in Sjögren's syndrome. J Rheumatol 35:2229-2235, 2008

II Spaan M, **Porola P**, Laine M, Rozman B, Azuma M, Konttinen YT. Healthy human salivary glands contain a DHEA-S processing intracrine machinery, which is deranged in primary Sjögren's syndrome. J Cell Mol Med 13(7): 1261-1270, 2009

III Virkki LM, **Porola P**, Forsblad D'Elia H, Valtysdottir S, Kvist G, Waltbrand E, Solovieva SA, Konttinen YT. Dehydroepiandrosterone (DHEA) in Severe Fatigue in DHEA-deficient Patients with Primary Sjögren's Syndrome. Arthritis Care Res (Hoboken) 15; 62(1):118-124, 2010

IV **Porola P**, Straub RH, Virkki LM, Konttinen YT, Nordström D. Failure of oral DHEA treatment to increase local salivary androgen outputs of female patients with Sjögren's syndrome. Scand J Rheumatol, *submitted*

V **Porola P**, Laine M, Virtanen I, Pöllänen R, Przybyla BD, Konttinen YT. Androgens and integrins in salivary glands in Sjögren's syndrome. J Rheumatol 37(6): 1181-1187, 2010

Publication V has been used in the dissertation of Mikael Laine (Pathological Changes at the Target Tissue Level in Sjögren's Syndrome and their Effect on the Exocrine Function of the Salivary Glands, University of Helsinki 2010).

2. ABBREVIATIONS

3-α-diol	androstane-3α,17β-diol
3α-diol-G	androstane 3α,17β-diol-glucuronide
ACTH	adrenocorticotropic hormone
ADTG	androsterone-glucuronide
AR	androgen receptor
BAFF	B cell activating factor
BM	basement membrane
CBG	corticosteroid-binding globulin
cDNA	complementary deoxyribonucleic acid
CRH	corticotropin-releasing hormone
CRISP-3	cysteine-rich secretory protein
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DMARD	disease-modifying antirheumatic drug
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FSH	follicle stimulating hormone
GC	germinal center
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HRT	hormone replecement therapy
HSD	hydroxysteroid dehydrogenase
HSE	hydroxysteroid epimerase
IFN	interferon
IL	interleukin
IRF5	interferon regulatory factor 5
LH	luteinizing hormone

MFI	Multidimensional Fatigue Inventory questionnaire
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
OATP	organic anion transporting polypeptide
P450c17	steroid 17-α-hydroxylase/17,20 lyase (Cytochrome P450 17A1)
P450scc	mitochondrial cholesterol side-chain cleavage enzyme (Cytochrome P450 11A1)
PBS	phosphate buffered saline
pSS	primary Sjögren's syndrome
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
SHBG	sex-hormone binding globulin
SSc	systemic sclerosis
sSS	secondary Sjögren's syndrome
STAT4	signal transducer and activator of transcription 4
STS	steroid sulfatase
SULT	sulfotransferase
TGF	transforming growth factor β
TLR	Toll like receptor
TNF-α	tumor necrosis factor

3. ABSTRACT

Sjögren's syndrome (SS) is a rheumatic autoimmune disease with yet unknown etiology. SS is characterized by sicca symptoms caused by involvement of the exocrine glands, mainly dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). In SS glands secretory acinar cell function is impaired and acinar cells are lost leading to diminished secretion of the glands, and a coexistent ductal cell hyperplasia is seen. SS is a strongly female dominant disease (9/10 of patients being women) and the diagnosis usually takes place between the ages of 40 and 50 years.

In humans and other primates the synthesis of sex steroids takes place in the gonads as well as in peripheral tissues such as adipose tissue, prostate and breast tissue. This local synthesis, called intracrine synthesis occurs from adrenal gland derived pro-hormones dehydroepiandrosterone (DHEA) and androstenedione by the intracrine steroidogenic enzymatic machinery and can lead to synthesis of either androgens or estrogens according to local needs. Aging affects hormonal production, especially in women. After menopause the gonadal estrogen synthesis ceases and simultaneously the synthesis of the pro-hormones from the adrenal cortex decreases. SS diagnosis is often done at the time of these hormonal alterations. Accordingly, the late onset of SS combined with the gender-bias encourage to believe that sex steroids have a role in the etiology and progression of SS.

Before this thesis primary observations about low serum DHEA levels in SS patients had been published. Our earlier studies showed diminished expression and secretion of androgen-regulated biomarker cysteine-rich secretory protein 3 (CRISP-3) all over SS salivary glands, also in areas remote of lymphocyte infiltrates. This led to the assumption that hormonal imbalance, more specifically depletion of immunoprotecting androgens, could be one of the factors behind SS. We hypothesized that SS patients suffer from androgen depletion both systemically but in particular locally in salivary glands. We believed that this hormonal imbalance in the exocrine glands could lead to structural changes and further to functional defects in SS salivary glands. Accordingly, the aim of these studies was to clarify the role of sex steroids in SS and thus to elucidate some of the so far obscure etiopathology of the disease. The main analytical methods used were quantitive polymerase chain reaction (qPCR)

on messenger RNA (mRNA) level and immunofluoresence stainings, enzyme-linked immunosorbent assay (ELISA) and Western blotting on protein level.

We confirmed our hypothesis and showed that SS patients have lower concentrations of DHEA, testosterone and dihydrotestosterone (DHT) both systemically in serum and locally in saliva compared with age- and sex-matched healthy controls. We especially focused on local hormonal enviroment in salivary glands and demonstrated for the first time that healthy salivary glands contain complete enzymatic machinery for local intracrine sex steroid synthesis. In SS salivary glands this machinery was found to be defective and, in a subgroup of patients, practically non-functional suggesting differencies between different SS patients. DHEA replacement therapy was found unbeneficial for SS patients, possibly due to the local defect in DHEA processing.

After discovering the androgen depletion in SS the effect of this defect on salivary glands was studied. We found that in salivary gland cells and healthy labial salivary glands androgens regulate integrin subunits α1 and α2, important for communication, differentiation and function of acinar cells. On the contrary, in SS salivary glands DHEA failed to upregulate these signaling molecules. This finding highlights the importance of local androgen environment and intracrine processing for the function and welfare of salivary glands.

To conclude, this study shows that patients with SS are androgen depleted both systemically and locally in salivary glands. SS patients also have a defective local intracrine sex steroid synthesizing enzymatic machinery further impairing the local androgen depletion. We also showed a tentative connection of androgen depletion and faulty intracrine activity with structural changes seen in SS salivary glands, thus linking hormonal imbalance to acinar loss and impaired glandular structure and function. In this study we have clarified some etiopathogenetic mechanisms of SS, which have thus far remained obscure, by showing the eventual importance of sex steroid imbalance in the onset and progression of SS.

4. REVIEW OF THE LITERATURE

4.1. Sjögren's syndrome

SS is a chronic autoimmune rheumatic disease, which occurs almost exclusively in women with the female-to-male ratio being 9-to-1 and the majority of the diagnoses being made in the fourth or fifth decade of life. According to the current consensus SS is always characterized by focal adenitis and/or serum antibodies of the SS-A/Ro and SS-B/La type. In addition to these obligatory autoimmune features the syndrome is characterized by dysfunction and atrophy of the acinar cells of the exocrine glands, particularly salivary and lacrimal glands, leading to diminished secretory capacity of the glands and further to main symptomatic features of the syndrome, oral and lacrimal dryness (xerostomia and keratoconjunctivitis sicca, respectively) (Vitali *et al*., 2002). As a result of mucosal dryness, patients with SS are affected by local complications such as caries and oral candidosis more often than healthy controls (Soto-Rojas *et al*., 1998). Although the disease is mainly targeting salivary and lacrimal glands, all other exocrine glands of the human can be involved as well. Besides oral and ocular symptoms, Sjögren's syndrome can also lead to manifestations in upper airways and genitals**,** for example (Freeman *et al*., 2005; Haga *et al*., 2005). SS can exist either in primary or secondary form (pSS and sSS, respectively). pSS evolves without co-existing autoimmune rheumatic disease, whereas in secondary form there is a verification of another underlying autoimmune disease in addition to and preceding SS, the highest degree of overlap occuring with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis (SSc) with RA being the most common one (Vitali *et al*., 2002; Ramos-Casals *et al*., 2007).

In addition to the glandular sicca symptoms, patients with SS suffer from diverse non-exocrine systemic and visceral symptoms and complications demonstrating that this is a systemic autoimmune disease.

The most disturbing and probably most disabling non-exocrine symptom is chronic fatigue. In Multidimensional Fatigue Inventory questionnaire (MFI-20; Smets *et al.,* 1995) measuring general, physical and mental fatigue, reduced motivation and reduced activity SS patients scored 15.6 in a scale from 4 (no fatigue) to 20 (maximal fatigue) compared with a score of

8.2 in healthy age-matched women (Barendregt *et al*., 1998). In some patients fatigue may have an apparent reason, such as impaired quality of sleep as a result of aches and pains, disturbing xerostomia and polydipsia and nocturia which may wake up the patient several times each night and lead to non-restorative sleep and early morning fatigue. Some other patients may suffer from anemia, actively ongoing inflammation and acute phase response or autoimmune thyroiditis, in which cases the patient usually wakes up refreshed but develops early afternoon fatigue when they run out of energy.
Psychiatric symptoms of SS such as anxiety and depression are closely connected with fatigue. Therefore, health-related quality of life in patients with SS is lower than in general population (Baturone *et al*., 2009; Segal *et al*., 2009). However, in most cases the reason and pathogenesis of fatigue in SS patients are still unknown.

Systemic manifestations of SS can be divided into non-visceral (fatigue, arthralgia, myalgia, Raynaud's phenomenon or "white fingers", skin) and visceral symptoms (lungs, heart, kidneys, gastrointestinal tract, endocrine, nervous system) (Fox, 2005). In addition, patients with SS have an increased, even over 40 times higher than normal, risk for lymphoma, with patients with pSS having a higher risk than those with the sSS (Kauppi *et al*., 1997; Davidson *et al*., 1999; Theander *et al*., 2006). Certain risk factors such as mixed cryoglobulinemia, palpable purpura, low C4 and peripheral neuropathy predict the development of malignant lymphoma in SS patients. Mortality in SS is slightly increased compared to general population and is mostly explained by remarkably high incident of lymphoma (Voulgarelis and Skopouli, 2007; Voulgarelis and Tzioufas, 2010).

Due to varying diagnosis criteria over time and in different countries the estimates of the prevalence of SS have fluctuated. However, according to studies made using the current, widely accepted classification criteria (Vitali *et al*., 2002) the prevalence of pSS has been estimasted to be around 0.1-0.6 %, 0.15 % or 0.72 % (Bowman *et al*., 2004; Andrianakos *et al*., 2003; Kabasakal *et al*., 2006, respectively). sSS is estimated to occur in 4-31 %, 9-19 % and 14-20 % of patients with RA, SLE and SSc, respectively (Andonopoulos *et al*., 1987; Nossent and Swaak 1998; Gilboe *et al*., 2001; Manoussakis *et al*, 2004; Avouac *et al*., 2006). SS is estimated to be one of the three most common autoimmune diseases together with RA and SLE (Pillemer *et al*., 2001). The criteria used in epidemiological studies affects the outcome and thus, due to different criteria, the prevalence of SS is difficult to estimate.

4.1.1. Diagnosis and assessment of Sjögren's syndrome

American- European consensus classification criteria are nowadays often used for the diagnosis of SS (Vitali *et al*., 2002). For a long time the diagnosis of SS was based on different criteria in Europe (Vitali *et al*., 1993) and USA (Fox *et al*., 1986) and classification criteria accepted in both continents were not available until the announcement of the current criteria in 2002 when the European criteria from 1993 were somewhat modified. Modifications were made considering the classification criteria, in particular the immunological features, which now are always required for the diagnosis based on the consensus criteria. Also classification of primary and secondary forms of the disease was sharpened in the American-European classification criteria (Vitali *et al*., 2002).

The current American- European classification criteria define the ocular and oral symptoms, ocular signs and salivary gland involvement (Table 1). To confirm the diagnosis of pSS, the patient has to fulfill either four of the six criteria with IV (histopathology) and/or VI (serology) being positive or three of the four objective criteria (III-VI). In addition to the sicca symptoms and signs, a distinguishing feature of SS is the autoimmune nature of the disease. As mentioned above, unlike the pervious criteria, the current criteria always require an indication of autoimmunity in form of either autoantibodies (SS-A or SS-B) in serum or focal sialadenitis in labial salivary gland biopsy (focus score, the number of clusters of 50 or more lymphocytes per 4 mm^2, must be $\geq$ 1). SS-A/Ro antibodies occur more commonly among patients with SS (with 30 to 70 % of patients being positive) than SS-B/La antibodies, which are less frequent (20 to 40 %) (Wahren-Herlenius *et al*., 1999). Diagnosis of sSS requires a verification of another underlying autoimmune disease according to currently valid criteria and a presence of either ocular or oral symptoms (I and II) added with two of the items III, IV or V (Table 1) (Vitali *et al*., 2002). Some characteristics not included in the classification criteria are the above mentioned female dominance, late onset of the disease, other sicca symptoms besides those of mouth and eyes and general and visceral symptoms and signs.

Table 1. Revised American-European classification criteria for SS (modified from Vitali *et al*., 2002).

Symptom	Definition
I Ocular symptoms	A positive response to at least one of the following: Continuous feeling of dry eyes for more than 3 months OR recurrent feeling of sand in the eyes OR use of tear substitutes more than 3 times a day
II Oral symptoms	A positive response to at least one of the following: Continuous feeling of dry mouth for more than 3 months OR persistently swollen salivary glands as an adult OR frequent drinking of liquids to aid in swallowing
III Ocular signs	A positive response to at least one of the following: Schirmer's test ≤ 5 mm in 5 minutes OR Rose bengal score ≥ 4 according to van Bijsterveld's scoring system
IV Histopathology	Focus score ≥ 1 in minor salivary glands, defined as a number of lymphocytic foci per 4 mm^2 of glandular tissue
V Salivary gland involvement	A positive result in at least one of the following diagnostic tests: 1) Unstimulated whole salivary flow ≤ 1.5 ml in 15 minutes OR 2) diffuse sialectasias in the parotid gland without evidence of obstruction in the major ducts OR 3) delayed uptake, reduced concentration or delayed excretion of tracer shown by salivary scintigraphy
VI Autoantibodies	Presence in serum of one or both of the following autoantibodies: SS-A/Ro, SS-B/La

In addition to inclusion criteria, the American- European classification criteria provide exclusion criteria for SS, because SS needs to be distinguished from other conditions with specified cause affecting exocrine glands, such as human immunodeficiency virus and human hepatitis C virus infections. Besides these viral infections exculsion criteria include the use of anticholinergic drugs, past head and neck radiation treatment affecting the exocrine glands,

pre-existing lymphoma, sarcoidosis and graft versus host disease. The list of exclusion criteria, based on a list by Fox *et al.* in a previous Californian (US) criteria for SS (Fox *et al*., 1986) was improved in the new criteria. The following exclusions were added to the list: past radiation treatment of the head and neck, hepatitis C virus infection and the sentence "use of anticholinergic drugs" instead of "use of antidepressant, antihypertensive, parasympatholytic drugs and neuroleptic agents". Additionally, sialoadenosis was deleted from the new exculsion criteria (Vitali *et al*., 2002).

Besides making the diagnosis of SS somewhat complicated, the variable diagnosis criteria have also caused underdiagnosis and diversity in the estimations of the prevalence of SS and its various extraocular and extraoral features. In addition, the diagnosis has been made difficult by the fact that the SS diagnosis, like the diagnosis of many other rheumatic diseases, is based on the demonstration of several different manifestations instead of a single major feature. In retrospect, in individual patients it has often taken many years for the whole syndrome complex to evolve. The classification of SS in either primary or secondary form further complicates the diagnosis because it may in individual cases be difficult to differentiate between an underlying autoimmune disease and autoimmune visceral manifestations of the syndrome, not least because many autoimmune diseases are preceded by even many years long subclinical phase.

Assessment of SS is challenging as well. The potentially reversible inflammation can lead to permanent damage in the affected glands and the distinction between SS activity and damage is thus difficult but essential. Other requirements of a valid assessment index include validity, reliability and sensitivity to change. Although separate criteria are used for the evaluation of SS activity and damage, the major weakness in the assessment of SS is the lack of an objective and standardised consensus criteria (Campar and Isenberg, 2010).

As a consequence of the above-mentioned problems, diagnosis and assessment of SS is still challencing, especially in the beginning of the illness when the clinical picture is mild. A better understanding of etiology and pathomechanism of SS, incorporation of screening strategies and development of standardised assessment criteria are required for more reliable diagnosis and assessment.

4.1.2. Etiopathogenesis of Sjögren's syndrome

Etiology

The primary reason for SS is still obscure despite intensive research. Different factors suggested to contribute to the etiopathogenesis of SS include genetic background, epigenetics, dysfunction of autonomic nervous system and different environmental factors such as viral infections and hormonal factors (table 2). The epidemiology of pSS is strongly biased by both chronobiology and by gender, which suggests a role for sex steroids in the etiology of the syndrome. Before these thesis works, no studies had been reported in the literature on local salivary sex steroid levels in SS.

Table 2. Factors suggested to contribute to SS etiology.

Factor	References
Genetic factors HLA Interleukin-10 IRF5 STAT4	Loiseau *et al*., 2001; Tzioufas *et al*., 2002 Hulkkonen *et al*., 2001 Miceli-Richard *et al*., 2007; Nordmark *et al*., 2009 Korman *et al*., 2008; Nordmark *et al*., 2009
Epigenetics	Stea *et al*., 2007
Viruses Epstein-Barr virus Human T-cell leukemia virus	Horiushi *et al*., 1999 Terada *et al*., 1994
Dysfunction of autonomic nervous system	Santavirta *et al*., 1997
Hormonal factors	Valtysdottir *et al*., 2001

Pathogenesis

Since no clear-cut singular reason has been found to SS, its pathogenesis is believed to be multifactorial. One of the most typical pathological findings in SS glands are local lymphocyte infiltrates (table 3). Another characteristic SS-feature is the presence of autoantibodies (table 3). SS-A/Ro and SS-B/La autoantibodies are directed towards a Ro-ribonucleoprotein, the function of which is largely obscure but is believed to be in regulation of translation and post-translational modification (Fabini *et al*., 2000; Jonsson *et al*., 2007b). Besides the presence of glandular infiltrates and SS-autoantibodies, which are also included in the diagnostic criteria of the disease (Vitali *et al*., 2002), SS pathogenesis includes many other

factors. Levels of many proinflammatory cytokines and chemokines are increased in patients with SS (table 3). Besides infiltrating lymphocytes, also epithelial cells are considered to produce pro-inflammatory cytokines as part of the inflammatory response (Garcia-Carrasco *et al*., 2006).

Other contributors in SS pathology are Toll like receptors (TLRs), which are considered to activate salivary gland epithelial cells and stimulate B cells (Gottenberg *et al*., 2006; Kawakami *et al*., 2007; Spachidou *et al*., 2007), and glandular epithelial cells themselves, which can have an active role in the pathogenesis of SS via unveiling of intracellular autoantigens and secretion of cytokines (Voulgarelis and Tzioufas, 2010) (Table 3). Characteristic findings in SS glands also include germinal centers (GC), in which the positive selection of autoreactive B-cells and thus the induction of autoimmunity are proposed to happen in GCs (Vinuesa *et al*., 2009).

Table 3. Factors suggested to contribute to SS pathogenesis.

Factor		References
Lymphocyte infiltrates	T cells B cells Macrophages Dendritic cells	Katsifis *et al*., 2007; Manoussakis *et al*., 2007; Youinou, 2008; Katsifis *et al*., 2009;
Autoantibodies	SS-A/Ro SS-B/La α-fodrin M3 receptor	Borda *et al*., 1996; Fabini *et al*., 2000; Ulbricht *et al*., 2003; Jonsson *et al*., 2007b
Cytokines	TNF-α TGF-β IL-1, IL-6, IL-8, IL-17 IFN-α, IFN-γ BAFF	Koski *et al*., 1995; Pflugfelder *et al*., 1999; Xanthou *et al*., 2001; Salomonsson *et al*., 2002; Szodoray *et al*., 2003; Båve *et al*., 2005; Katsifis et al., 2007; Katsifis *et al*., 2009
Toll-like receptors	TLR2, -3, -4, -8 and -9	Gottenberg *et al*., 2006; Kawakami *et al*., 2007; Spachidou *et al*., 2007
Epithelial cells		Voulgarelis and Tzioufas, 2010
Germinal centers	Correlation with higher focus scores and productin of autoantibodies	Salomonsson *et al*., 2003; Jonsson *et al*., 2007a Vinuesa *et al*., 2009

One possible route for the pathogenesis of SS has been suggested to follow a path where a triggering factor in genetically predisposed individuals leads to invasion of the infiltrating

cells to exocrine glands. This invasion further causes apoptosis and/or necrosis of the glandular cells, reveals cryptic epitopes and is followed by the production of autoantibodies. Consequently, this further interferes with the glandular function. TLRs and increased secretion of proinflammatory cytokines may also contribute to the pathological process (Garcia-Carrasco *et al*., 2006). However, a definite pathway for the pathogenesis of SS has not been defined yet. The mechanism of the acinar atrophy and glandular destruction in SS is considered multifactorial and is believed to involve several cell types (inflammatory cells, epithelial cells) and molecules (cytokines, autoantibodies, TLRs and enzymes). Inflammation and deteriorated function of the exocrine glands may also represent separate processes in the pathogenesis of SS.

4.1.3. Treatment of Sjögren's syndrome

Medical treatment of SS can be divided in two major divisions: local treatment for sicca symptoms and systemic treatment for extra-glandular manifestations. Most patients can manage with only local treatment (artificials tears, ocular ointments, topical non-steroidal anti-inflammatory drugs and chewing gum, sweets, artificial saliva for dry eyes and/or mouth). Local estrogens are used for genital dryness and epithelial atrophy (Jonsson *et al*., 2007b; Ng and Isenberg, 2008).

Systemic treatment includes the use of secretagogues, steroidal and non-steroidal anti-inflammatory agents and disease-modifying and biologial agents. Pilocarpine and cevimeline, which are acetylcholine receptor agonists, have been shown to be beneficial in the treatment of oral and ocular dryness (Mavragani *et al*., 2006). Systemic corticosteroids are mainly used in the treatment of severe extra-glandular features, such as nervous system and renal manifestations. Patients with arthralgias or arthritis receive non-steroidal anti-inflammatory drugs. Treatment of extra-glandular changes and high systemic inflammatory activity also includes disease modifying and/or immunosuppressive drugs (hydroxychloroquinone, methotrexate). Of extra-glandular symptoms, the treatment of fatigue of patients with SS is most challenging. Recently there have been reports about positive effects of rituximab therapy on fatigue (Dass *et al*., 2008; Alcântara *et al*., 2009).

Many novel candidates such as nizatidine (a H2 receptor antagonist), mizoribine (a suppressor of lymphocyte proliferation) and rebamipide (an anti-inflammatory mucosal protective agent) are promising as xerostomia and keratoconjunctivitis sicca therapies (Kapoor, 2009). Of new

potential medications in particular biological agents have been in focus of recent studies. Despite of the elementary role of TNF-α in inflammation and tissue damage in SS, anti-TNF therapies (infliximab, etanercept) have in controlled clinical trials proven unhelpful (Mariette *et al*., 2004; Ramos-Casals *et al*., 2010). Similarly, IFN-α proved to be only as effective as placebo, measured by stimulated whole saliva flow and oral dryness (Cummins *et al*., 2003). On the contrary, primary results from the use of B-cell targeting rituximab show some promise in some patients in both the treatment of SS and prevention of SS-associated lymphoma, although the adverse effects (infusion reactions, neutropenia, human antichimeric antibodies) can be significant. B-cell depletion therapy has also proven to improve stimulated and unstimulated whole saliva flow rate in a subset of patients with baseline salivary flow >0.1 ml/min. However, most studies show no changes in the objective measures of dryness with the rituximab treatment (Pijpe *et al*., 2005; Isaksen *et al*., 2008; Alcântara *et al*., 2009; Meijer *et al*., 2010). Especially extra-glandular manifestations have improved with rituximab, particularly in early stages of the disease (Dass *et al*., 2008; Alcântara *et al*., 2009). Also epratuzumab (anti CD-22 antibody) might be effective (Steinfeld *et al*., 2006). The mechanism of action of B-cell-targeted therapy might function via blocking antigen presentation by B cells to T cells or by preventing the secretion of B cell derived cytokines and autoantibodies. Anti-CD20 -therapy also works on memory B cells clustering in SS salivary glands, which do not proliferate and thus do not respond to therapies such as methotrexate mainly affecting proliferating cells. Future biological therapies may also target molecules like BAFF or IFN.

Lack of consensus assessment criteria for SS, along with obscure etiology and disease process, complicates the interpretation and comparison of clinical trials studying new SS medications and thus complicates the treatment of SS. Accordingly, evidence-based therapy of SS is mainly limited to treatment of sicca symptoms.

4.2. Sex steroids

Sex steroids, androgens, estrogens and prostagens, are steroid hormones synthesized by a specialized endocrine tissue from where they are secreted into blood. From bloodstream they access their target cells and bind to specific receptors. Sex steroid –receptor complexes act by regulating gene expression of their target genes. On the other hand, in human and other

primates sex steroids can also be synthesized locally in the peripheral tissues from the adrenal gland derived pro-hormones DHEA, DHEA sulfate (DHEA-S) and androstenedione (Labrie *et al*., 2005). The main androgens produced in human are testosterone and DHT, an androgen with the greatest affinity towards the androgen receptor, whereas 17-β-estradiol is the most potent estrogen of the human body.

Sex steroids affect both primary and secondary sexual characteristics. Physiological functions of estrogens include among others maintenance of bone mass, regulation of insulin responsiveness and regulation of lipoprotein synthesis in addition to regulating the development of female sex organs and characteristics. In men, estrogens have been found to be fundamental to fusion of epiphyses as well as to maintenance of bone mass (Nelson and Bulun, 2001). Androgens, for their part, have a pivotal role in male reproductive function. They also regulate pubertal development and contribute to secondary sexual characteristics in males (Quigley *et al*., 1995; Gelmann 2002). Testosterone, as well as androstenedione, also serves as precursors that can be aromatized into estrogens (Walters *et al*., 2008). In addition to their roles in reproductive processes, both estrogens and androgens are thus considered to have general metabolic roles in both sexes (Simpson, 2003).

4.2.1. Synthesis and metabolism of sex steroids

Sex steroids are cholesterol derivates. Both estrogens and androgens are synthesized from DHEA, which is derived from cholesterol (Figure 1). Synthesis of active sex steroids from DHEA goes through androstenediol and androstenedione with DHT and 17-β-estradiol as final products of the androgen and estrogen pathways, respectively. Gonads, testes and ovaries, are the primary synthesizers of sex steroids. Ovarian follicles and corpus luteum secrete mainly estrogens and progesterone, but also androgens are released. The primary source of androgens is the Leydig cells of the testes (Kroboth *et al*., 1999; Sanderson, 2006).

In addition to synthesis in the gonads, humans synthesize sex steroids locally in peripheral tissues. This local synthesis stems from the unique feature of primates: they possess a reticular zone in their adrenal glands that secretes large amounts of so-called prohormones DHEA, DHEA-S and androstenedione. The cortex of the human adrenal glands consists of three layers, all with different functions. The outermost layer, *zona glomerulosa* and the middle layer *zona fasciculata* produce mineralocorticoids and glucocorticoids, respectively.

The innermost layer, *zona reticularis*, is the location of the synthesis of DHEA, DHEA-S and androstenedione (Hornsby, 2004). The synthesis of DHEA from cholesterol includes two steps: first, cholesterol is converted to pregnenolone after side chain cleavage by mitochondrial cholesterol side-chain cleavage enzyme ($P450_{scc}$) in mitochondria, and subsequently pregnenolone is processed into DHEA through the action of steroid 17-α-hydroxylase/17,20 lyase (P450c17) (Kroboth *et al*., 1999). DHEA can further be converted into DHEA-S, the most common circulating steroid hormone in human, by sulfotransferase (Figure 1) (Labrie, 2003).

Adrenal gland derived precursor hormones constitute a large reservoir of substrate that can be processed into active estrogens or androgens extragonadally by steroidogenic enzymes according to local needs in different peripheral tissues (Labrie, 1991). These intracrinologically produced sex steroids function in the same cells in which they are produced without further release into blood (Nestler *et al*., 1988). Classical intracrine tissues include tissues such as prostata and mammary gland. Many other tissues, such as adipose tissue and bone, have been shown to produce sex steroids locally as well (Simpson, 2003).

The first step in the transformation of DHEA into androstenedione (4-dione), a step common to synthesis of both androgens and estrogens, is catalyzed by 3β-hydroxysteroid dehydrogenase (3β-oli-dehydrogenase $\Delta^{4,5}$ –isomerase) (3-β-HSD). There are multiple isomers of this enzyme enabling the tissue- and cell specific regulation of the enzymatic activity. Also 17β-OH- steroid dehydrogenase (17-β-HSD) enzymes exist in multiple types, of which some catalyze the reductive reaction in the synthesis pathway and some work in the opposite direction thus inactivating androgens and estrogens. 5-α-reductase catalyzes the 5-α-reduction of 4-dione to androstanedione and testosterone to DHT, the most active androgen. The synthesis of estrogens, namely 17-β-estradiol, is catalyzed by aromatase (Labrie *et al*., 2005) (Figure 2). Processing of the adrenal precursor steroids into active androgens and/or estrogens in peripheral target tissues is depended on the expression levels of steroidogenic and sex steroid metabolizing enzymes in these tissues.

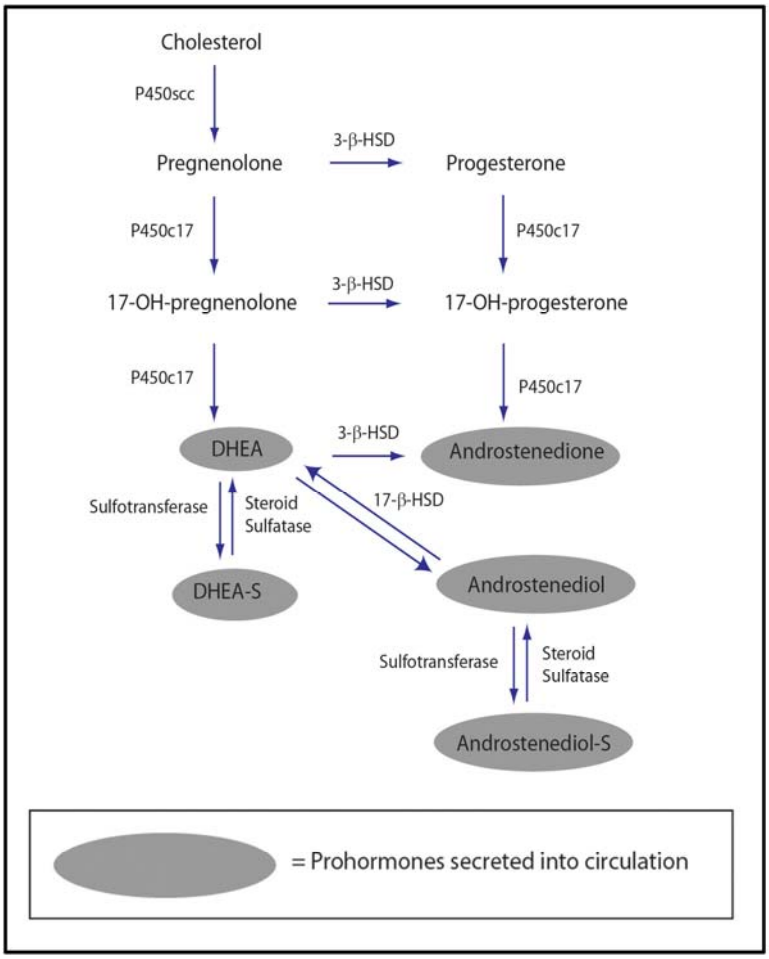

Figure 1. Synthesis of prohormones dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S) and androstenedione from cholesterol in *zona reticularis* of the adrenal glands. P450scc = mitochondrial cholesterol side-chain cleavage enzyme (Cytochrome P450 11A1), P450c17 = Steroid 17-α-hydroxylase/17,20 lyase (Cytochrome P450 17A1), 3-β-HSD = 3β-hydroxysteroid dehydrogenase (3β-oli-dehydrogenase $\Delta^{4,5}$ –isomerase), 17-β-HSD = 17-β-hydroxysteroid dehydrogenase (Modified from Miller, 2009).

Local production of sex steroids is of extreme importance in human and 90 % of DHEA and 98 % of DHEA-S in circulation are produced by the adrenal cortex (Knochenhauer and Azziz, 2001; Labrie, 2003). Approximately 50 % of total androgens in the prostate of an adult man are synthesized in an intracrine fashion. In women the local intracrine synthesis is even more important: 75 % and nearly 100 % of peripheral estrogens in pre- and postmenopausal women, respectively, are synthesized locally (Belanger *et al.*, 1986; Labrie, 1991). Total

androgen production, estimated from the measurement of the conjugated DHT metabolites which are considered to represent the most reliable parameters of the total androgen pool, in women represents two-thirds of the corresponding values in men and the majority of these androgens originate from the peripheral production (Labrie *et al*., 1997a).

Sex steroids are metabolized first by phase I enzymes, after which phase II enzymes act on these metabolites forming conjugates that are usually more polar and thus more easily secreted by the kidneys. In the case of DHT, phase I metabolites include androsterone, epiandrosterone, androstane-3α,17β-diol (3-α-diol) and androstane-3 β,17β-diol, which are formed through the action of many 3-α/β-HSD and 17- β-HSD isoforms. For final metabolism of androgens phase II enzymes, such as UDP-glucuronosyltransferases, convert phase I metabolites further into conjugates, like androstane-3α,17β-diol-glucuronide (3-α-diol-G) and androsterone-glucuronide (ADTG), the two major phase II metabolites of DHT (Figure 2).
In peripheral tissues, DHT is considered to be metabolized into conjugates locally before leaving the tissue and appearing in the circulation. Thus, as mentioned above, serum levels of these metabolites are considered to reflect the androgen status in the peripheral tissues and by controlling the metabolizing enzymes the androgen levels of these tissues can be regulated (Bélanger *et al*., 2003).

Regulation of sex steroid synthesis

Gonadal production of sex steroids is regulated by luteinizing hormone and follicle stimulating hormone (LH and FSH, respectively). Secretion of androgens from the Leydig cells is mainly regulated by LH (Franchimont, 1983). Sex steroid production in the ovaries is explained by the two-cell theory according to which LH induces the production of androgens from cholesterol in the theca cells while FSH stimulates the conversion of theca cell derived androgens to estrogens in the granulosa cells (Falck, 1959, Sanderson, 2006).

Synthesis of sex steroid precursors from the adrenal glands is in part regulated by adrenocorticotropic hormone (ACTH) secreted from the pituitary gland. The hypothalamic corticotropin-releasing hormone (CRH) stimulates the secretion of ACTH, which further stimulates adrenal cortex to secrete gluco- and mineralocorticosteroids and adrenal precursor hormones (Kroboth *et al*., 1999). Also local factors in the adrenal cortex, such as cytokines derived from immune cells (macrophages, lymphocytes) and adrenal cells themselves

modulate the synthesis of adrenal prohormones (Ehrhart-Bornstein *et al.*, 1998). The degree of the peripheral synthesis of sex steroids depends on the amounts of adrenal gland derived precursor hormones as well as on the expression and function of the steroidogenic enzymes.

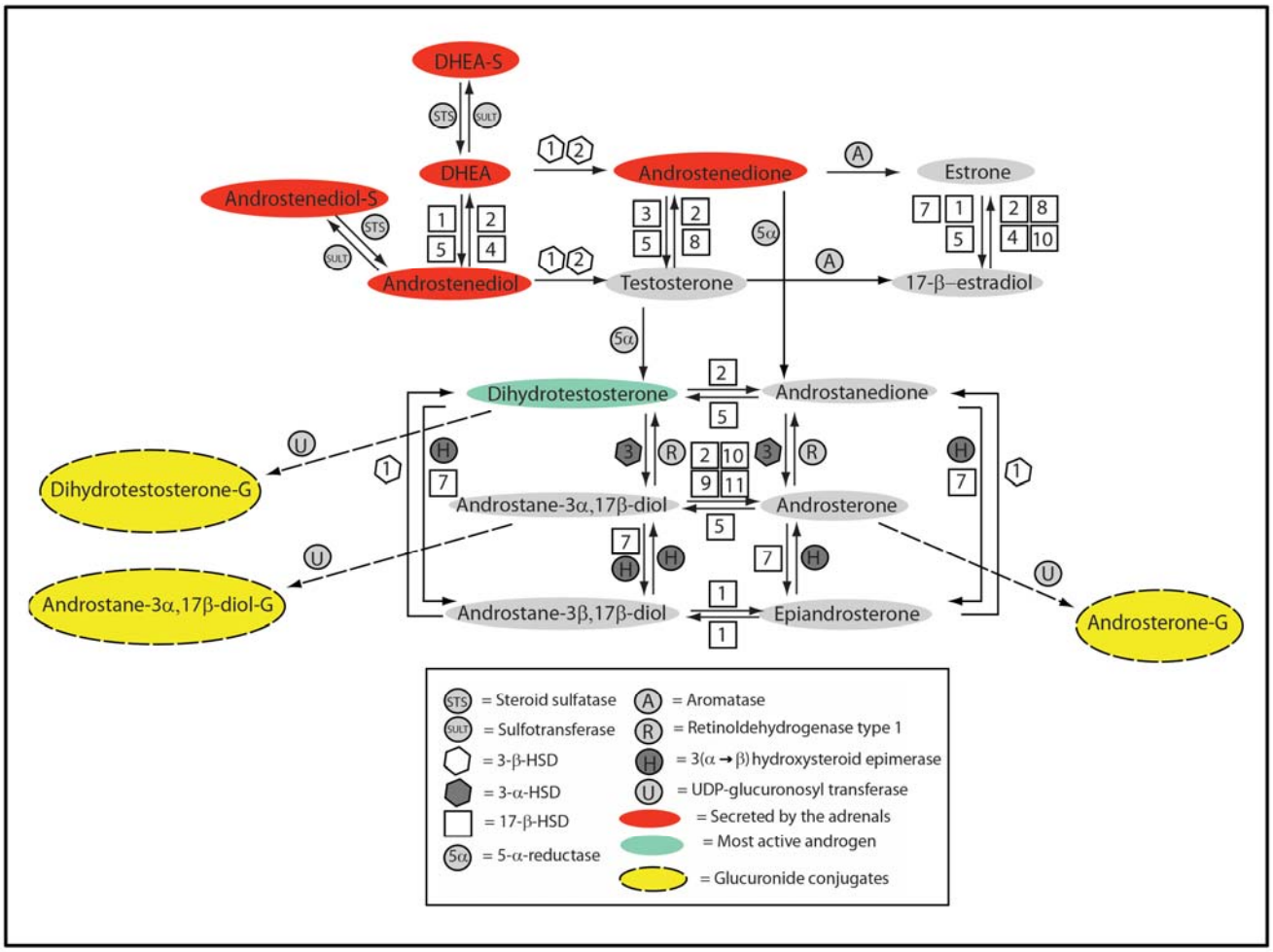

Figure 2. Peripheral androgen metabolism. Adrenal gland derived prohormones are converted to most active androgen, dihydrotestosterone, which is further metabolized and finally inactivated by UDP-glucuronosyltransferases. Subtypes of each HSD enzyme is marked with a number. DHEA = dehydroepiandrosterone, DHEA-S = dehydroepiandrosterone sulfate, G = glucuronide, HSD = hydroxysteroid dehydrogenase (Modified from Bélanger *et al.*, 2003 and Konttinen *et al.*, 2010) .

4.2.2. Mode of action of sex steroids

Since sex steroids are hydrophobic, they circulate mostly bound to carrier proteins, which can be either general (with low affinity for steroids) or specific to steroids (with higher affinity). Only approximately 2 % of the total sex steroid amount in the circulation exists as a free fraction (Dunn *et al*., 1981). Albumin is the most common general carrier protein, whereas liver-derived sex-hormone binding globulin (SHBG) and corticosteroid-binding globulin (CBG) are specific carriers. Most serum androgens and estrogens are carried by SHBG and to a minor extent by albumin (Hammond *et al*., 2003; Michels and Hoppe, 2008). According to the free hormone hypothesis steroid hormones are thought to enter their target cells only from the free portion and not from the protein-bound portion. Thus, intracellular hormone concentrations have traditionally been considered to reflect the concentration of the corresponding free hormone in plasma (Mendel, 1992).

To carry out their functions, steroids have been considered to diffuse through the plasma membrane and bind to their intracellular, mostly cytoplasmic receptors. Both androgens and estrogens act in the same manner: they bind to their receptors, translocate to the nucleus and mediate changes in the transcription of their target genes (Figure 3). Androgen and estrogen receptors (AR and ER, respectively) belong to the family of nuclear receptors and are ligand-dependent transcriptional factors. The structure of AR and ER consists of three domains with different functions: a function-contributing variable N-terminal domain, ligand binding domain, which binds the steroid ligand and DNA binding domain which binds to target DNA (Evans, 1988; Gelmann, 2002).

In addition to the classical mode of action through their nuclear receptors, androgens and estrogens can operate in rapid non-genomic manner and have an influence faster, within seconds to minutes either by binding with G-protein coupled receptors (membrane androgen receptors or sex-hormone binding globulin receptors) or by binding directly to their target proteins (ion-channels and transporters) (Falkenstein *et al*., 2000; Michels and Hoppe, 2008). Megalin is one receptor mediating non-conventional actions of sex steroids. It is able to bind SHBG-steroid complex thus enabling the entry of also that fraction of sex steroids bound to SHBG, previously considered “inactive” according to the free hormone hypothesis, into tissues (Hammes *et al*., 2005). Whether a steroid will induce a rapid or a genomic signal

depends on the steroid in question, on the target cell and on the location of the receptor within a cell.

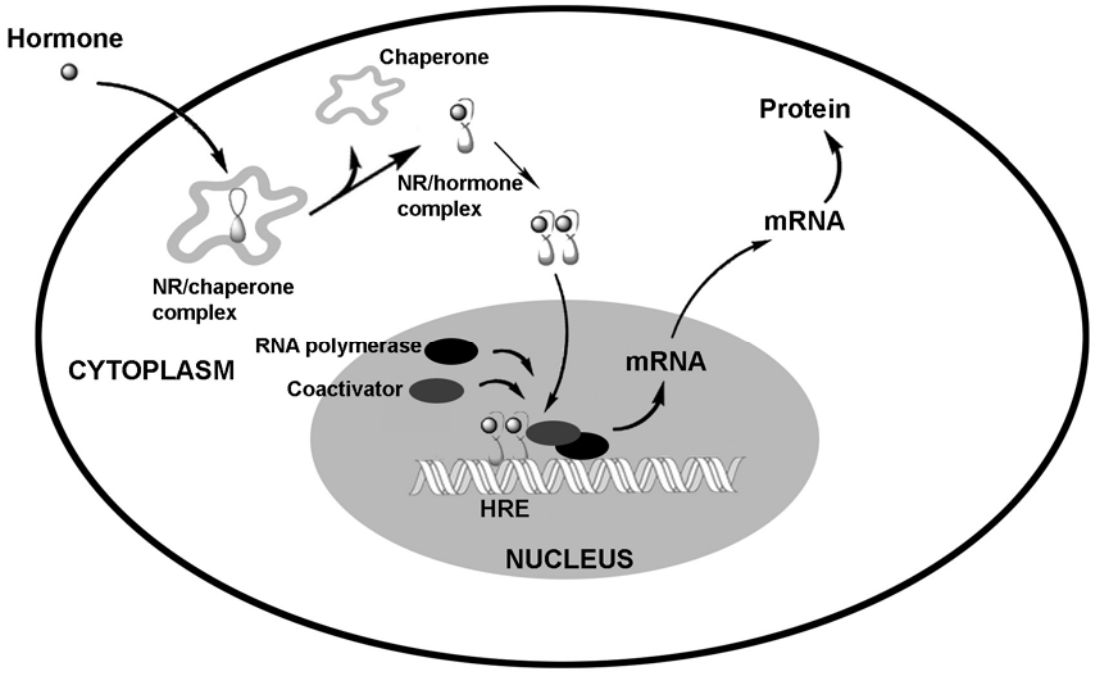

Figure 3. Classical mode of action of sex steroids. Hormones enter the cell by passive diffusion and bind to the chaperone-bound nuclear receptor (NR). The binding of hormone to its receptor releases the chaperone and leads to receptor dimerisation. NR/hormone dimers translocate to the nucleus and bind to the hormone responsive element (HRE) on their target gene together with coactivators and RNA polymerase. The outcome of the hormonal effect is an alteration in the target gene expression.

There are controversial opinions about the existence of specific receptors for DHEA. Specific receptors for DHEA have been suggested to exist in T-cells and vascular endothelial cells (Meikle *et al*., 1992; Liu and Dillon, 2004). Additionally, it has been shown that in endothelial cells, blocking of ER or AR did not inhibit DHEA-induced effects. However, these effects were reversed by inhibition of Gi/o-proteins, suggesting involvement of a specific plasma membrane, Gi-protein bound DHEA receptor other than ER or AR and a non-genomic action of DHEA (Williams *et al*., 2004; Liu *et al*., 2008).

4.2.3. Sex steroid imbalance and immunity

Sex steroids have remarkable effects on the immune system. Estrogens stimulate immunoglobulin production in peripheral blood mononuclear cells and enhance humoral immunity (Kanda and Tamaki, 1999). Estrogens and their metabolites also have a positive effect on the secretion of proinflammatory cytokines (Janele *et al*., 2006). Contrary to the role of estrogens, testosterone was reported to inhibit secretion of proinflammatory cytokines such as TNF and IFN-γ (Janele *et al*., 2006). Testosterone also inhibited the production of antibodies by peripheral blood mononuclear cells both in normal persons and patients with SLE (Kanda *et al*., 1996; Kanda *et al*., 1997) and is suggested to inhibit IL-1 secretion by macrophages (Cutolo *et al*., 1995). Further support to the anti-inflammatory roles of androgens is given by the study showing that androgen therapy is effective in the treatment of RA (Cutolo *et al*., 1991; Booji *et al*., 1996).

However, there are results showing opposite roles for estrogens and androgens. Besides stimulating antibody formation, estrogens have been suggested to be anti-inflammatory (Carlsten, 2005). Also, the effect of estrogens on immunity has been reported to depend on the concentration: low and high concentrations of estradiol in chronic inflammatory diseases are thought to be pro- and anti-inflammatory, respectively (Cutolo and Wilder, 2000). Testosterone has been reported to have proapoptotic effects on activated macrophages, where as 17-β-estradiol had opposite effects (Cutolo *et al*., 2005). Furthermore, estrogen deficiency as a result of aromatase depletion has been suggested to cause a SS like disease in mice (Shim *et al*., 2004) and estrogen deficiency has been reported to induce apoptosis resticted to tubuloacinar cells, which may induce α-fodrin autoantibodies and a SS-like condition in mice.

Besides the immunomodulatory role of active estrogens and androgens, pro-hormones DHEA and androstenedione are reported to be immunomodulatory *per se* or by virtue of their metabolites. Studies suggest them to enhance the immune function and protect mice from microbial infections (Padgett *et al*., 1995). Additionally, DHEA supplementation has been shown to decrease T helper cells and increase natural killer cells in postmenopausal women as well as to increase monocytes, B-cells and T-cell activation in older men (Casson *et al*., 1993; Khorram *et al*., 1997). Consequently, DHEA has been proposed to participate in the activation of the immune system. DHEA has also been shown to inhibit formation of murine autoantibodies in SLE mouse model (Lucas *et al*., 1985).

4.3. Salivary glands

Salivary glands are branched saliva producing glands belonging to exocrine glands, which also include pancreas, liver and gall bladder in the digestive tract. The secretions from these glands are directed to the lumen of the digestive tract, where they operate mostly in digesting food.

4.3.1. Anatomy and histology of salivary glands

There are two types of salivary glands in humans and other mammals: three pairs of main salivary glands (submandibular, sublingual and parotid gland located under the mandible, under the tongue and between the upper and lower jaw in the back of the mouth, respectively) and a large number of minor salivary glands with a short salivary duct, located directly under the oral mucosa. Salivary glands share a common structure with saliva producing acini in the end of the branches, ducts, which deliver the secreted saliva from the acinus into oral cavity and connective tissue surrounding the gland (Tucker, 2007). Ducts can be divided into intercalated ducts located next to the acini, striated ducts and finally excretory ducts leading to the oral cavity (Figure 4). Ducts of the parotid gland are long and open opposite to the upper second molar. Saliva from the submandibular and sublingual glands is drained under the tongue and in the floor of the mouth, respectively (Ogawa, 2003; Tucker, 2007). Minor salivary glands have similar structure with acini and ducts. These glands are small and also ducts of the minor glands are short.

Basement membrane in salivary glands

As every tissue, also salivary glands are surrounded by extracellular matrix (ECM), which is an extracellular part of tissues providing structural support to cells, having also other functions (Figure 4).

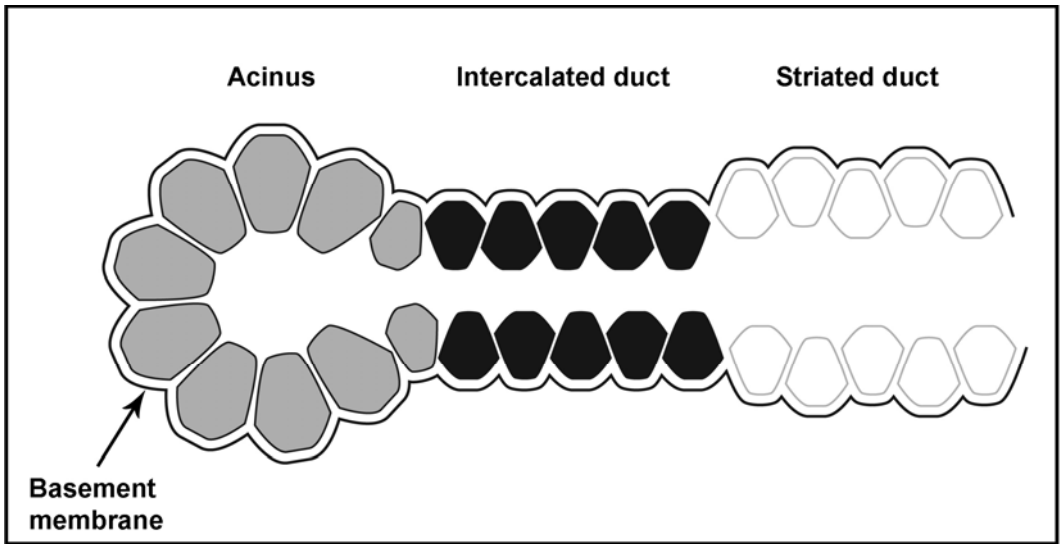

Figure 4. Schematic picture of a salivary gland. Saliva secreting acinus consists of acinar cells and salivary duct of intercelated ductal cells located next to the acinus and striated ductal cells located closer to the oral cavity. Basement membrane surrounds the gland.

Basement membrane (BM) is a specialized part of the ECM directly underlying epithelial and endothelial cells and separating them from the interstitial stroma. BM consists mostly of laminin and collagen type IV connected together by nidogen-1 and perlecan (LeBleu *et al*., 2007). BM does not only act passively in supporting cells but has also a functional role. BM serves as a storage of information and growth factors and influences and modifies cellular morphogenesis, differentiation, growth and functionality through outside-in signalling in salivary glands as in other glandular tissues (Ekblom *et al*., 1998; LeBleu *et al*., 2007).

Laminins represent the major protein components of BM. They are large heterotrimeric glycoproteins composed of one of five α, three β and three γ chains, bound together by disulfide bonds to form usually cross-like structures with three short and one long arm (Aumailley *et al*., 2005). In salivary glands, ductal and acinar parts of the BM express different laminin compositions. Laminin α2 chain is considered characteristic for the myoepithelial cells (Strassburger *et al*., 1998), whereas α1 is seen in specifically acinar BMs apparently in contact with secretory acinar cells (Laine *et al*., 2004). Laminin α3, α5, β1, γ1 and γ2 chains are found both in ductal and acinar compartments. Laminins represent probably the major instrument of communication between BM and epithelial cells. They store information and upon binding to cellular receptors, such as integrins and dystroglycan, deliver

messages from BM to cells. Especially integrins have a pivotal role in the interaction of cells with the BM. Except for laminins, integrins serve as receptors for other ECM proteins and cell surface adhesion molecules. They are expressed in different heterodimeric combinations of α and β subunits with different ligand specificities (Lourenço and Kapas, 2005). Like laminins, also integrins have different expression patterns in different parts of salivary glands with α1 and α2 subunits representing acinar integris and α3, α6, β1 and β4 subunits being expressed both in acinar and ductal compartments (Laine *et al*., 2008).

Interactions between BM constituent laminins and their cellular integrin receptors are crucial for salivary glands. They operate in branching morphogenesis and the functional differentiation of salivary gland cells (Kadoya *et al*., 1997; Durbeej *et al*., 2001: Lam *et al*., 2005; Lourenço and Kapas, 2005; Szlávik *et al*., 2008). Interactions between integrins and BM ligands lead to a variety of signalling responses such as changes in calcium influx, cytoplasmic pH, phospolipase activity and protein phosphorylation in the cell (Lafrenie and Yamada, 1998). In addition, integrins also function as biosensors of BM composition and control the survival or programmed death of the cell (Stupack and Cheresh, 2002).

4.3.2. Saliva

Saliva is an exocrine fluid consisting mainly (approximately 99%) of water. Other components of saliva include a variety of electrolytes (sodium, potassium, calcium, magnesium, bicarbonate, phosphates), proteins such as immunoglobulins, antimicrobial peptides, enzymes and mucins and nitrogenous products like ammonia and urea. Whole saliva is a mixture of secretions from both major and minor salivary glands added with gingival fluid containing bacteria and food debris, epithelial and blood cells as well as traces of medicines and chemical products (Ogawa, 2003; Tucker, 2007).

Main functions of saliva

Saliva has multiple functions with its three main roles being lubrication and protection of oral tissues, antimicrobial properties and buffering of acid attacks. By lubricating oral tissues saliva facilitates the movements of lips and tongue thus aiding swallowing and speaking. The protective role of saliva includes inhibiting carcinogens and plaque-derived proteolytic and other hydrolytic enzymes from causing damage. The lubricating and protecting function relies

in particular on mucins, which comprise approximately a quarter of the salivary proteins. Mucins are complex glycoproteins existing in salivary glands predominantly in two forms, the high-molecular-weight (oligomeric) MG1 and the low-molecular-weight (monomeric) MG2 (Saari *et al*., 1997; Zalewska *et al*., 2000; Humphrey and Williamson, 2001).

Mucins also participate in the antimicrobial role of saliva. They offer a major binding site for microorganisms entering the mouth aggregating them and thus inhibit microorganisms from reaching oral tissues. Other antimicrobial agents of saliva include molecules (lactoferrin, lysozyme, peroxidase) inhibiting bacterial growth and destroying them, proteins and peptides aggregating bacteria and secretory immunoglobulins, mainly secretory IgA blocking adhesion of bacteria to host tissues. In addition to IgA, phagocytosis-enhancing IgG and IgM exist in saliva. Additionally, the flushing effect of saliva from mouth to gut is as such an important protective factor against microbes (Tenovuo 1998; Humphrey and Williamson, 2001).

The buffering capacity of saliva, too, serves to protect the mouth from microbes. The main component of the salivary buffering system is bicarbonate which diffuses into plaque and neutralizes acids. Furthermore, bicarbonate generates ammonia form amines, thus adding the neutralizing effect. In addition to the bicarbonate system, phosphates and the protein system buffer saliva. The buffering capacity of saliva also serves as a factor in maintenance of tooth integrity by facilitating remineralization and inhibiting caries progression. Other functions of saliva include enhancing taste and digesting food (Humphrey and Williamson, 2001).

Secretion of saliva

Salivary glands are innervated by both parasympathetic and sympathetic nerves. With major glands, the secretion of the watery and organic component of saliva is generated by parasympathetic and sympathetic stimulation, respectively (Garrett, 1987). A salivary center in the medulla controls the secretion and can be stimulated by mechanical, gustatory and olfactory stimuli. In addition, psychic factors and certain medications affect the secretion. Main salivary glands are responsible for the saliva production and they produce approximately 90 % of the saliva with 20 % coming from the parotid, 65 % from the submandibular and 7 to 8 % from the sublingual gland. Minor salivary glands produce the remaining 10 %. The relative contribution of different salivary glands changes between unstimulated and stimulated salivary flow so that minor salivary glands produce

proportionally more during rest whereas the parotid gland is responsible for approximately 50 % of the stimulated salivary production (Humphrey and Williamson, 2001). In addition to their volume main salivary glands also differ in the type of saliva they produce. Acini can be divided into serous and mucous: serous acinar cells secrete watery secretion with a large amount of proteins whereas mucous cells secrete mucous saliva with large glycoproteins called mucins. Parotid gland is a serous gland whereas the sublingual glands are mucous. Submandibular gland is a seromucous gland producing saliva with both proteins and mucins.

Stimulation of myoepithelial cells by the autonomous nerves results in the contraction of these cells surrounding acini and some parts of the ducts generating movement of the secretion (Tucker 2007). Upon contraction, the protein- or carbohydrate -rich granular content of serous and mucous acinar cells, respectively, is secreted in parallel with water and electrolytes. This secretion, primary saliva, is consequently forced out of the acini into the ducts, which collect and transport the secretion to the oral cavity. Striated and excretory ductal cells also modify primary saliva: they reabsorb Na^+ and Cl^- and secrete low amounts of K^+ and HCO_3^-. Due to the low permeability of the ducts to water, the secondary saliva secreted to the oral cavity is hypotonic compared to primary saliva. Intercalated ductal cells do not take part in processing of primary saliva (Humphrey and Williamson, 2001; Ogawa, 2003; Tucker, 2007).

The molecular mechanisms leading to the production of primary saliva involve activity of four ion channels. In the resting phase, basolateral ion pumps Na^+,K^+,$2Cl^-$ –cotransporter and Na^+/K^+ ATPase pump Cl^- and K^+ into acinar cell. The activation of either cholinergic or α-adrenergic receptors leads to increased release of Ca^{2+} from endoplasmic reticulum into the cytosol. Ca^{2+} further activates Ca^{2+} sensitive basolateral K^+ and apical Cl^- channels leading to leakage of these ions into circulation and to the lumen of the acinus, respectively. Release of K^+ hypopolarizes/sensitizes the acinar cells. Na^+ follows Cl^-, and water is consequently secreted according to the osmotic gradient into the lumen through apical aquaporin channels and between the cells. After the stimulation, concentration of Ca^{2+} decreases followed by elevation of Na^+ concentration due to the activity of both Na^+-H^+ exchanger and Na^+ -coupled transport of Cl^- and K^+. This flow of ions induces the uptake of water to the cell. Also reuptake of K^+ and Cl^- follow (Nauntofte, 1992).

The average amount of whole saliva secreted is between 0.5 and 1.5 liters a day with a remarkable variability between individuals. Any amount of unstimulated salivary secretion above 0.1 ml/min is normal and salivary flow less than that is considered hyposalivation. For stimulated saliva the corresponding limit is 0.7 ml/min.

Aging as such does not cause hypofunction of the salivary glands. Instead, systemic diseases and increased use of medicines in the elderly people are believed to induce reduced salivary flow as a side effect (Baum, 1981; Parvinen and Larmas, 1982). However, in aging individuals the acinar glandular tissue of salivary glands is replaced with fatty and fibrous tissue leading to reduction of acinar components and further to diminished salivary gland function (Nagler, 2004). Indeed, results implying a reductive role for aging in salivary flow have been presented (Yeh *et al*., 1998; Streckfus *et al*., 2002).

5. AIMS OF THE STUDY

Regardless of its relatively high prevalence and intensive research, the pathogenesis of SS is still obscure. The female dominance, late onset and targeting of the disease into all exocrine glands encouraged us to hypothesize that sex steroids, especially androgens, play an important role in the emergence and progression of SS. The aim of these studies was to clarify some roles of sex steroids in the etiology and pathogenesis of SS with the following specific objectives:

1. To test our hypothesis about androgen defect in SS by examining the androgen and estrogen levels in healthy controls and in patients with SS both systemically in serum and locally in saliva

2. To investigate the local DHEA-S processing intracrine enzymatic machinery *in vitro* and *in vivo* in healthy and SS salivary glands by studying its expression and function and the capability of oral DHEA replacement therapy to normalize systemic and, more importantly, local salivary levels of androgens in SS patients

3. To study if oral DHEA benefits SS patients with severe fatigue and low serum DHEA-S concentration

4. To study the effect of androgens on the expression of laminin-111 and integrins α1β1 and α2β1, which are important for salivary gland acinar cell differentiation and signalling in order to study the connection between androgen deficiency and acinar atrophy seen in SS salivary glands

6. MATERIALS AND METHODS

6.1. Cell cultures

6.1.1. Cell line (I, V)

HSG is an irradiated neoplastic intercalated ductal cell line derived from the human submandibular gland (Shirasuna *et al*., 1981). The cell line can be induced to express an acinar phenotype by culturing it on basement-membrane mimicing, laminin α1 chain containing Matrigel (BD Biosciences, San Jose, CA) (Royce *et al*., 1993). Cells were cultured in DMEM/F-12 Nut Mix medium (Gibco BRL, Grand Island, NY) supplemented with 10 % fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO_2-in-air at + 37°C.

6.1.2. Cell stimulations (I, V)

For stimulations, HSG cells were grown in 6- or 24-well-plates with number of cells per well being 3 x 10^5 or 5.5 x 10^4, respectively. Cells grown in wells without or with Matrigel coating (BD Biosciences, San Jose, CA) (ductal and acinar phenotypes, respectively) were stimulated with 1, 10 or 100 µM DHEA (Fluka, St. Louis, MO; Sigma-Aldrich, St.Louis, MO) (I, V), 1 µM testosterone (Sigma-Aldrich, St.Louis, MO) (V), 1, 10, 100 or 1000 nM DHT (Fluka, St. Louis, MO; Sigma-Aldrich, St.Louis, MO) (I, V) or 100 nM 17-β-estradiol (Sigma-Aldrich, St.Louis, MO) (V). Stimulations were performed in serum-free media for 72 hours. Every stimulation was done in triplicate or multifold. After the stimulations, culture media were collected and stored in -70 ºC.

Inhibitor of 5-α-reductase types I and II (Dutasteride; GlaxoSmithKline, Middlesex, UK) or II (Finasteride; Sigma-Aldrich, St. Louis, MO) was added at the same time as the hormones (I).

6.2. Patients and samples (I-V)

Table 4. Subjects of the studies. In healthy controls the diagnosis of primary SS was refuted using the American-European consensus criteria (Vitali *et al*., 2002).

Study	Study I	Study II	Study III	Study IV	Study V
No of subjects (Blood and saliva samples)	43 pSS / 15 healthy		107 pSS	12 pSS	
No of subjects (Fatigue assesment)			107 pSS		
No of subjects (LSG tissue samples)	2 healthy	IF: 4 pSS / 4 healthy Tissue culture: 3 pSS / 4 healthy			3 pSS / 4 healthy
Age	33-78 (pSS) / 32-73 (healthy)	44-65 (pSS) / 17-57 (healthy)	36-78 years (pSS)	44-70 years (pSS)	44-65 (pSS) / 17-57 (healthy)
Inclusion criteria	pSS (Vitali *et al*., 2002)	pSS (Vitali *et al*., 2002)	pSS (Vitali *et al*., 2002) Low DHEA-S serum values Severe fatigue (MFI-20 general fatigue score≥14) (Smets *et al*., 1995)	pSS (Vitali *et al*., 2002) Low DHEA-S serum values	pSS (Vitali *et al*., 2002)
Exclusion criteria	Use of glucocorticoid, DMARDs, immuno-suppressive therapy		Age <18 years or >80 years, history of certain cancers, difficult acne, significant liver disease and previous stroke or known diathesis for thrombosis, prednisolone >10mg per day, pregnant, lactating women, fertile patients without adequate prevention	Age <18 years or >80 years, history of certain cancers, difficult acne, significant liver disease and previous stroke or known diathesis for thrombosis, prednisolone >10mg per day, pregnant, lactating women, fertile patients without adequate prevention	

6.3. Tissue cultures (I, II, V)

The samples were minced into pieces (approximately 2 mm^3), put into a 6-well-plates and left overnight in DMEM/F-12 medium (Gibco BRL, Grand Island, NY) containing 10 % fetal calf serum with 2mM L-glutamine, 1000 U/ml penicillin, 1 mg/ml streptomycin (10X) and Fungizone (2.5 µg/ml) (Gibco BRL, Grand Island, NY) solution. The next day, the media were changed to basal DMEM/F-12 media with 10 % serum-stripped fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 0,1 mg/ml streptomycin (1X solution) and 2.5 µg/ml Fungizone.

6.4. Design of the clinical dehydroepiandrosterone trial (III)

pSS-patients (n=107) were randomized into two treatment groups at the beginning the study either to receive DHEA 50 mg *q.d.* or placebo. The first treatmetn period (4 months) was followed by a 1 month long wash-out period. After that, cross-over was performed after which the group that had received DHEA in the first treatment period now received placebo during the second treatment period (4 months) and *vice versa.* Patients visited researchers 1) in the beginning of the study (0 months), 2) after the first treatment period (at 4 months), 3) after the wash-out period (at 5 months) and 4) after the second treatment period (at 9 months). Compliance was estimated by counting the surplus tablets returned by patient and by measuring serum DHEA-S levels at the last visit of the patients (at 9 months).

6.5. Collection of saliva and serum samples (I, III-IV)

The blood samples were let to stand for 45 minutes and then centrifuged for 10 minutes (1500 x *g*). Serum was collected and stored at -20°C.

Unstimulated whole saliva was collected for 15 minutes and stimulated saliva for 5 minutes when chewing paraffin capsules. Saliva samples were centrifuged (10 minutes, 100 x *g*, +4ºC), proteinase inhibitor (Complete, Roche, Basel, Switzerland) was added and the samples were stored at -20°C.

6.6. Measurement of sex steroid concentrations (I, III-IV)

Serum levels of DHEA-S were measured with a radioimmunometric assay (Thermo, Waltham, MA) (I, III) or ELISA (IBL, Hamburg, Germany) (IV) and serum concentrations of DHEA, androstenedione, free testosterone, DHT, 17-β-estradiol and 3α-diol-G with ELISA kits (IBL, Hamburg, Germany) (I, IV).

Total salivary levels of DHEA-S, DHEA, androstenedione, testosterone, DHT, 17-β-estradiol and 3α-diol-G were measured with salivary enzyme immunoassays (Salimetrics, State College, PA; IBL, Hamburg, Germany) or ELISA kits (IBL, Hamburg, Germany) (I, IV). Frozen samples were melted on ice and centrifuged (15 minutes at 200 x *g*). The clear supernatant was used for the analysis.

6.7. Assesment of fatigue and health-related quality of life in patients with Sjögren's syndrome (III)

Fatigue was evaluated using a MFI-20 questionnaire. MFI-20 is a multidimensional self-report instrument measuring five dimensions of fatigue: general fatigue, physical fatigue, mental fatigue, reduced motivation and reduced activity (Smets *et al*., 1995). In our study the MFI-20 questionnaire also included a visual analogue scale (VAS), which measures the subjective global fatigue.

Health-related quality of life was measured either by the RAND-36 or SF-36 questionnaire (Hays *et al*., 1993; Sullivan *et al*., 1995; Persson *et al*., 1998; Sullivan *et al*., 1998) for Finnish and Swedish speaking patients, respectively. These questionnaires, however, are almost identical.

6.8. Measurement of serum autoantibodies (IV)

Serum autoantibodies (SmB, SmD, RNP-70k, RNP-A, RNP-C, SS-A/Ro52, SS-A/Ro60, SS-B/La, Cenp-B, Topo-I/Scl-70, Jo-1/HRS, Ribosomal RNP, histones) were analyzed in the serum of patients with SS before and after the DHEA replacement therapy. Semi-quantitative analysis was done with Inno-Lia ANA Update line-blotting strips (Innogenetics, Gent, Belgium) and results were interpretted as negative, borderline, weakly positive, moderately positive or strongly positive.

6.9. Analysis of messenger RNA expression levels (I-II, V)

6.9.1. RNA extraction and complementary DNA synthesis

Total RNA was extracted from cultured HSG cells with the Trizol method (Invitrogen, San Diego, CA), before which cells were washed with PBS. Before addition of the Trizol reagent, cells cultured on Matrigel were detached with Dispase (BD Biosciences, San Jose, CA) and washed with PBS-EDTA and PBS. From total RNA, messenger RNA was isolated with the Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway) (I).
Alternatively, RNA isolation from the cells was done with RNeasy Mini kit (Qiagen, Hilden, Germany) (V).
From tissue samples total RNA was isolated with High Pure RNA Tissue kit (Roche, Basel, Switzerland) (I, III, V).
The amount and quality of RNA isolated was measured spectrophotometically with Nanodrop (Thermo Fisher Scientific, Waltham, MA).

Complementary DNA (cDNA) synthesis for cellular RNAs was performed with SuperScript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) from 1500-2500 ng total RNA or 100 ng of mRNA.

6.9.2. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was done with LightCycler™ PCR mix and a LightCycler™ PCR instrument (Roche, Basel, Switzerland). Serial dilutions of the cloned target sequence were used as standards to determine the copy number of the amplicon in relation to house keeping genes. Porphobilinogen deaminase (PBGD) was used as housekeeping gene in cell experiments and β-actin in tissue experiments. The levels of the housekeeping genes were similar in every sample, diverging samples were excluded. All primers were designed so that the amplicon covered two exons to facilitate detection of possible DNA contaminations.
For detailed information about primers used in the studies, the reader is referred to the original publications.

6.10. Western blotting (I)

Cell culture media were concentrated using 10 kDa Centricon centrifugal filter device tubes (Millipore, Billerica, MA) and the total protein concentrations were measured with Bradford's method (Bradford, 1976).

Samples with equal amount of total protein (8.5 -30 µg) were boiled for 5 minutes in SDS-gel loading buffer. After electophoresis samples were blotted into Immobilon-P transfer membranes (Millipore, Billerica, MA) and blocked overnight with 3 % BSA in Tris-buffered saline in room temperature. Membranes were incubated in 3 µg/ml rabbit anti-human CRISP-3 antibody (Udby *et al.*, 2002) followed by alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch, Suffolk, UK). CRISP-3 protein was detected with Western Lightning CDP-Star Chemiluminescence Reagent (Perkin Elmer, Waltham, MA), followed by imaging of the membranes with ProXpress 2D Imaging System (Perkin Elmer, Waltham, MA). Results were quantified using Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

6.11. Immunofluorescent staining (II, V)

6.11.1. Study II

Labial salivary gland tissue samples were cut to 5 µm sections and fixed in 4% paraformaldehyde for 5 minutes. Unspecific binding sites were blocked with 1:10 diluted normal donkey serum (Jackson ImmunoResearch Europe Ltd., Suffolk, UK) for one hour at room temperature. Tissue sections were incubated in affinity purified primary antibodies specific for each intracrine enzyme (1) anti-human steroid sulfatase (STS) E.C.3.1.6.2 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml, 2) goat anti-human sulfotransferase (SULT) E.C.2.8.2.15 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml, 3) goat anti-human 3β-HSD E.C.1.1.1.145 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml, 4) goat anti-human 17β-HSD E.C.1.1.1.63 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml, 5) goat anti-human aromatase E.C.1.14.14.1 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml and 6) goat anti-human 5α-reductase E.C.1.3.99.5 IgG (Santa Cruz Biotechnology, Heidelberg, Germany), 20 µg/ml. After the primary antibody, sections were washed in 0.1 % Triton-X in phosphate buffered

saline (PBS), pH 7.5, followed by PBS alone. 10 μg/ml donkey anti-goat IgG (Alexa Fluor 488, Molecular Probes, Eugene, Oregon) was used as a secondary antibody for one hour at room temperature in dark. Next the sections were washed as above and sometimes counterstained with 0.014 mmol DAPI (Sigma-Aldrich, St.Louis, MO) nuclear stain in dH_2O for 5 minutes at room temperature in dark and again washed as above. Finally, sections were mounted with fluorescent mounting medium Vectashield (Vector Laboratories, Burlingame, CA). Non-immune, normal goat IgG was used instead of and at the same concentration as the primary specific IgG antibodies for staining controls.

6.11.2. Study V

Indirect immunofluorescence staining of the integrin type laminin receptors was performed with monoclonal IgG1 antibodies TS2/7 against integrin α1 subunit (Hemler *et al*., 1984) and 10G11 against integrin α2 subunit (Giltay *et al*., 1989). Cells were washed in 10 mM phosphate buffered, 150 mM saline, pH 7.4, containing 0.1% Triton X-100. Normal goat serum (X0907, Dako, Glostrup, Denmark) was used in the blocking step to diminish non-specific labelling. After incubation with primary antibodies cells were washed in Triton X-100 containing PBS. Bound antibodies were visualized using secondary goat anti-mouse IgG antibody (Alexa Fluor 488, Molecular Probes, Eugene, OR). Propidium iodide diluted 1:1000 in PBS was used for nuclear staining. After washes in Triton X-100 containing PBS coverslips were embedded in fluorescent mounting medium (Dako, Glostrup, Denmark) and examined under an Olympus AX70 (Tokyo, Japan) microscope coupled with a CCD camera (Olympus DP71). Control immunostainings were performed using non-immune IgG of the same sub-class as the primary antibodies at the same concentration as and instead of the primary specific antibodies and by using conjugated secondary antibodies alone.

6.12. Statistics (I, III-V)

Baseline demographics and disease characteristics were assessed using descriptive statistics. Differences in sex steroid levels between patients with SS and healthy controls were tested for significance with the t-test or Mann-Whitney U test (for normally and non-normally distributed data, respectively). Serum and salivary levels of hormones before and during DHEA treatment were compared using Wilcoxon signed rank test and correlations analyzed using the Spearman's test.

The method for cross-over trials by Altman (Altman, 1991) was used for the analysis of period effect, period-treatment interaction and treatment effect comparing DHEA and placebo. Intention-to-treat principle was adopted for the main analysis. Since the treatment was not anticipated to lead to permanent improvement of fatigue, a return to baseline was used as an estimate of the true state of the patient in case of discontinuation.

Integrin α1 and α2 mRNA levels without and with androgen and estrogen treatments were compared with Mann-Whitney test and the overall effects of stimulations with different androgen concentrations studied with Kruskall-Wallis's test.

All results are given as mean ± standard error of the mean values and median or percentage changes from the baseline. The level of significance was set at 0.05 and two-tailed levels of significance were used throughout the studies. Data were analyzed with SPSS statistical software, Version 13.0 (I), 14.0 (III) or 16.0 (IV-V) (SPSS, Chicago, IL).

7. RESULTS AND DISCUSSION

7.1. Systemic and local imbalance of sex steroid levels in patients with Sjögren's syndrome (I)

We hypothesized that patients with SS suffer from androgen depletion. Our hypothesis is supported by the facts that the majority of patients with SS are women and that the disease is usually diagnosed in patients around 50 years old, simultaneously with decreases in systemic levels of estrogens and adrenal steroids (menopause and adrenopause, respectively). To test our hypothesis we studied the systemic serum and local salivary hormonal status of SS patients and healthy controls.

7.1.1. Systemic sex steroids in Sjögren's syndrome

Subnormal DHEA(-S) levels in patients with SS had been reported before the thesis studies (Valtysdottir *et al*., 2001; Sullivan *et al*., 2003; Laine *et al.,* 2007). Like SS, many other autoimmune rheumatic diseases such as RA and SLE are female dominant (Beeson, 1994). Lower DHEA(-S) levels have been observed also in RA and SLE and patients with rheumatic autoimmune diseases are generally considered androgen deficient (Fehér and Fehér, 1984; Lahita *et al*., 1987; Spector *et al.*, 1988; Hedman *et al*., 1989; Stafford *et al*., 2000; Sullivan *et al*., 2003; Tengstrand *et al*., 2003; Imrich *et al*., 2005). On the other hand, not all studies have supported these observations (Cutolo *et al*., 1986; Brennan *et al*., 2003; Cevik *et al*., 2004).

Our hypothesis about the androgen depletion was based on the female dominance, late diagnosis and the above-mentioned DHEA(-S) depletion in SS. In addition, salivary glands are sexually dimorphic organs and considered a target tissue of androgens with androgen receptor localized to the nuclei of acinar and a majority of ductal cells (Laine *et al*., 1993; Treister *et al*., 2005). As a systemic factor androgen depletion could also explain targeting of SS into all exocrine glands of the body. Additionally, the inflammation-reducing effect of androgen replacement therapy in salivary (and lacrimal) glands of female SS mice model (Sato and Sullivan, 1994) further supports our theory.

An androgen deficiency of also other androgens besides DHEA was observed in pSS patients. We demonstrated that patients with pSS have significantly lower serum concentrations of DHEA-S and DHT compared to age- and gender-matched healthy controls. Also systemic concentrations of testosterone were decreased (Figure 5).

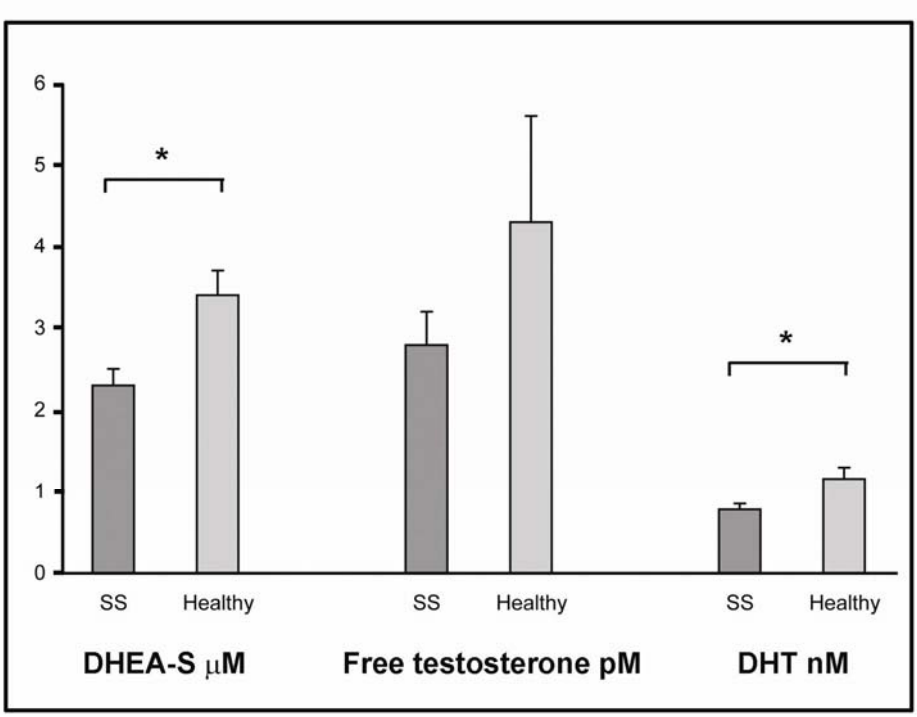

Figure 5. Serum concentrations of androgens in patients with Sjögren's syndrome (SS) (n=43) vs. age- and sex-matched healthy controls (n=15). DHEA-S = dehydroepiandrosterone sulfate, DHT = dihydrotestosterone. * = $p \leq 0.005$.

Interestingly, our group and others have found that serum testosterone levels differ only little between SS patients and healthy controls (Valtysdottir *et al*., 2001; Taiym *et al*., 2004). Testosterone is an "intermediate" between DHEA-S and DHT, both of which are decreased in SS, so the minor decrease in testosterone levels could suggest that the conversion of testosterone to DHT is ineffective in SS. However, this could also simply be due to the relatively wide variation in testosterone values.

Results showing no decreases in serum androgen levels in SS patients (Brennan *et al*., 2003) might be explained by medication. A significant proportion of both patients and healthy controls (31% and 47%, respectively) in this study used hormone replacement therapy (HRT).

After deletion of these subjects from the analysis, the number of healthy controls was very small (n=8), which could have affected the results. Also the concentrations of sex steroids were generally notably low in both groups. This could possibly be due to the use of corticosteroids, which was not mentioned. Additionally, some studies show no differences in the sex steroid concentrations of RA patients (Cutolo *et al*., 1986, Cevik *et al*., 2004). If low serum DHEA(-S) concentrations play a role for the pathogenesis of pSS, low serum DHEA(-S) concentrations in RA and SLE could play a role in the pathomechanisms of sSS. However, because pSS patients have not been studied prior to the disease outbreak, it is also possible that the low systemic DHEA(-S) concentrations are secondary to autoimmune-inflammation.

We also demonstrated that contrary to decresed androgen levels, systemic levels of 17-β-estradiol were increased in patients with pSS compared to healthy controls (325.6 ± 57.3 pM vs. 123 ± 41.9 pM, p=0.008). Accordingly, SS patients have a lowered androgen/estrogen ratio. This is in agreement with some previous studies showing increased concentrations of estradiol and/or its metabolites in SS and other autoimmune diseases (Lahita *et al*., 1979; Castagnetta *et al*., 2003; Tengstrand *et al*., 2003). However, similarly to androgen concentrations, the data concerning estrogen levels is conflicting: several studies have found no differencies or decreases in estrogen concentrations in patients with autoimmune rheumatic diseases compared with healthy controls (Masi *et al*., 1995; Sullivan *et al*., 2003; Taiym *et al*., 2004). These results suggest that the estrogen levels vary quite a lot depending on the age, menopause and other characteristics of the patients and controls.

7.1.2. Local sex steroids in Sjögren's syndrome salivary glands

We confirmed the hypothesis about local androgen deprivation in SS and showed that SS patients consequently have lower salivary secretion of all androgens measured (DHEA, testosterone, DHT) compared to age and sex matched healthy individuals (Figure 6). This finding fits with our earlier observation, which showed that local levels of DHEA and androgen-regulated CRISP-3 were decreased in pSS patients compared with healthy controls. CRISP-3 deficiency was seen all over the glands, also in areas remote of lymphocyte infiltrates implying that this phenomenon is not caused by infiltrating inflammatory cells (Laine *et al.*, 2007). Systemic estrogen excess extended to local level too, with salivary 17-β-estradiol concentrations in SS patients being 22.6 ± 7.5 pM compared to 21.0 ± 1.7 pM in healthy controls

(p=0.003), which reflects decreased local ratio of androgens in relation to estrogen in SS patients.

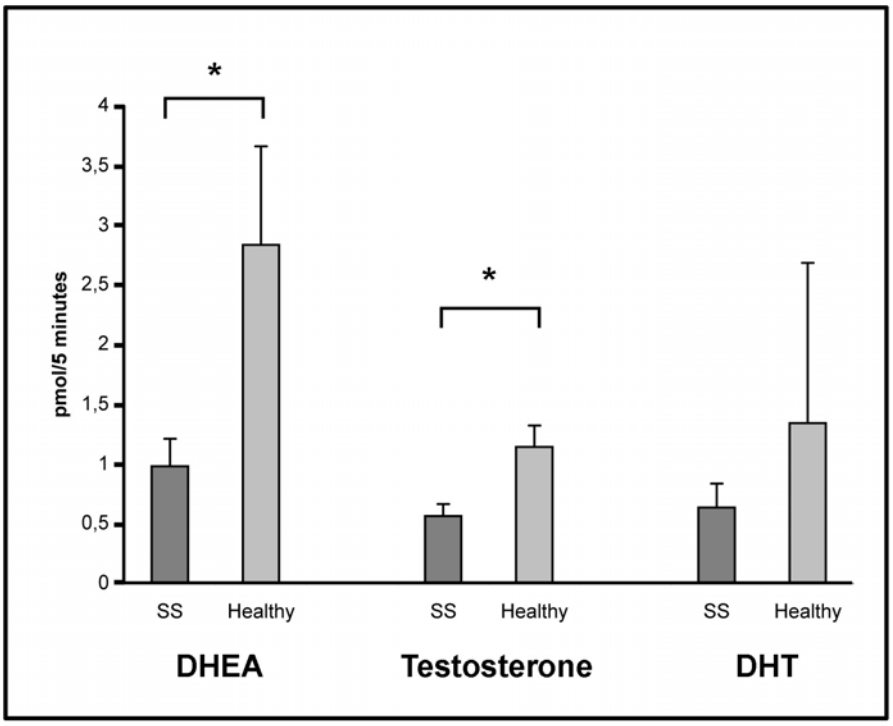

Figure 6. Salivary outputs of androgens in patients with Sjögren's syndrome (SS) (n=43) *vs*. age- and sex-matched healthy controls (n=15). DHEA = dehydroepiandrosterone, DHT = dihydrotestosterone. * = $p < 0.02$.

7.1.3. Eventual causes of systemic sex steroid imbalance in Sjögren's syndrome

Aging influences sex steroid production in humans. In women the production of estradiol from the gonads declines sharply at the time of menopause but the production of testosterone decreases only gradually (Simpson, 2003; Somboonporn, 2006). In men the age-related decrease in the production of androgens is mild and serum concentrations of estrogens either show a decline or stay at a steady state (Feldman *et al*., 2002).

Aging affects also adrenal production of pro-hormones. Serum concentrations of DHEA and DHEA-S reach their peak levels between 20 and 30 years, after which they decline steadily in both sexes. The most important decline in serum levels of adrenal pro-hormones thus precedes menopause and the most common age of SS diagnosis (Orentreich *et al*., 1984;

Labrie, 2003). Adrenopause may be due to age-related atrophy of *zona reticularis* (Pawlikowski, 2005) or decreased secretion of DHEA in response to ACTH, which has been reported to decrease with age (Ohashi *et al.*, 1986). Throughout the years, the concentrations of DHEA and DHEA-S are higher in men than in women, making women more vulnerable to disturbances in sex steroid balance.

Age-related changes lead to decreased levels of both androgens and estrogens. Accordingly, we believe that they are not solely responsible for the sex steroid imbalance in SS. Altered function of the hypothalamic-pituitary-adrenal (HPA) and/or hypothalamic-pituitary-gonadal (HPG) axis has been suggested in the context of autoimmune diseases and lower levels of ACTH have been measured in patients with SS. The pituitary and adrenal responses to ovine cCRH have been reported to be weakened in SS patients (Tanriverdi *et al*., 2003; Johnson *et al*., 2006). Also altered production of cytokines may influence the hormonal balance. Cytokine environment is disturbed in patients with SS as well as in other autoimmune diseases (Brennan *et al*., 1998; Pflugfelder *et al*., 1999; Aringer and Smolen, 2004). Inflammatory cytokines such as interleukins and TNF-α can down-regulate the synthesis of steroid hormones on the adrenal level as seen in chronic inflammations (Herrmann *et al*., 2002). In women with SS, the production of DHEA-S from the adrenals has been shown to be decreased although the production of cortisol was normal (Valtysdottir *et al.*, 2001). Besides cytokines, lymphocytes and macrophages in the adrenal glands could directly influence androgen-producing cells of *zona reticularis*.

7.2. Intracrine machinery in salivary glands (I-IV)

The unbalanced local hormone environment in SS salivary glands might be solely a reflection of the endocrine defect seen in SS. Alternatively, it can originate from a distinct local intracrine defect. As mentioned earlier, in primates sex steroids are synthesized locally in peripheral tissues by intracrine enzymes in addition to gonadal synthesis (Labrie *et al*., 2005). We wanted to study if salivary glands have such a local intracrine sex steroid synthesis machinery. In RA mixed synoviocytes such local sex steroid producing machinery has been shown (Castagnetta *et al*., 2003) but to our knowledge, before this thesis the complete intracrine potential of salivary glands had not been studied. Thus, our aim was to study the

expression and to some extent the function of DHEA processing steroidogenic enzymes in salivary glands, both of which we expected to be altered in SS.

7.2.1. Local intracrine enzymatic machinery has an organized architecture in healthy salivary glands

We demonstrated that salivary glands are intracrine organs. *In vitro* studies demonstrated that salivary gland cells convert DHEA to more active androgens. This was shown by studying the expression of androgen-regulated biomarker CRISP-3 (Laine *et al*., 2007), which was shown to be increased by both DHEA and DHT. However, the up-regulating effect caused by DHEA was abolished when the conversion of DHEA further to DHT was inhibited by incubating the cells with the potent type I and II 5-α-reductase inhibitor dutasteride. The same phenomenon was also suggested *in vivo* in healthy salivary glands, in which we observed salivary DHEA and testosterone not to correlate. In contrast, there was a correlation between salivary testosterone and DHT, as if a certain proportion of testosterone would always be converted to DHT. However, salivary DHEA levels did not show any correlation with the levels of 17-β-estradiol either in healthy or diseases glands. These results suggest effective conversion of DHEA towards androgens rather than estrogens in salivary glands.

We showed that in healthy salivary glands tubuloacinar epithelial cells contain a complete intracrine machinery for DHEA(-S) pro-hormone processing. These enzymes have an organized architecture with enzymes processing DHEA in basal parts of the cell. Enzymes catalyzing the synthesis of the most active steroids, 5-α-reductase (type I in salivary glands) and aromatase, are located in nucleus and apical membrane, respectively (Figure 7A). This organization is rational and seems to mirror the preferential site of action for each intracrine enzyme. SULT and STS process DHEA and DHEA-S and are thus localized near to the site of uptake of DHEA(-S) from the circulation, in the basal parts of the acinar cells. Their presence in the tubuloacinar epithelial cells is in line with previous reports showing cytoplasmic expression of these enzymes in prostate and ovarian epithelial cells (Okuda *et al*., 2001; He *et al*., 2004). Also the intermediate enzymes, 3- and 17-β-HSDs, were located in the basal cell parts. Androgen receptor is located in the nucleus of salivary acinar cells (Laine *et al*., 1993). The nuclear location of DHT synthesizing 5-α-reductase thus enables an effective and rapid local production and effects of DHT through the androgen receptor. Aromatase was found mostly in the apical membrane of the acinar cells suggesting export of estradiol out of

the acinar cells. Since estrogen receptors have been shown to situate mainly in salivary ductal cells and oral mucosal epithelium (Välimaa *et al*., 2004; Tsinti *et al*., 2009), we think that instead of intracrine utilization, estrogens are exported to saliva for further use in the ductal epithelial cells.

In contrast to acinar cells, in healthy ductal cells the organization of the intracrine enzymes was more diffuse. STS, SULT and 5-α-reductase were expressed more widely and found also in the cytoplasm. This was true also with 3- and 17-β-HSDs, which were also found near the apical plasmamembrane. Aromatase was in the ductal cells located in both the basal and apical plasma membrane. Thus, in salivary ductal cells the organization of the intracrine enzymes was not as strict as in acinar cells.

7.2.2. Dysfunctional intracrine machinery in Sjögren's syndrome salivary glands

We showed that the expression and localization of pro-hormone processing enzymes in SS salivary glands is disturbed. STS stained very weakly and was relatively diffuse and SULT was almost completely absent in SS acinar cells. The expression of 3- and 17-β-HSDs and 5-α-reductase was more diffuse and staining was observed also further in cytoplasm compared with the healthy acinar cells. In contrast to other steroidogenic enzymes, the expression pattern of aromatase was not changed in SS (Figure 7B). Thus, the disturbed expression in SS seems to affect especially the acinar cells and enzymes participating in the production of androgens for local intracrine use, especially that of DHT. This impaired subcellular compartmentalization of enzymes participating in the androgen production may explain the low local DHT outputs and previous results showing low salivary levels of androgen biomarker CRISP-3 in SS salivary glands (Laine *et al*., 2007). Furthermore, normal expression of aromatase might explain the higher outputs of estrogens in SS saliva. In earlier studies even increased aromatase expression in labial salivary glands from premenopausal SS patients has been shown (Onodera *et al.*, 1998). The activity of aromatase may be increased also in patients with SLE and RA (Folomeev *et al*., 1992; Castagnetta *et al*., 2003).

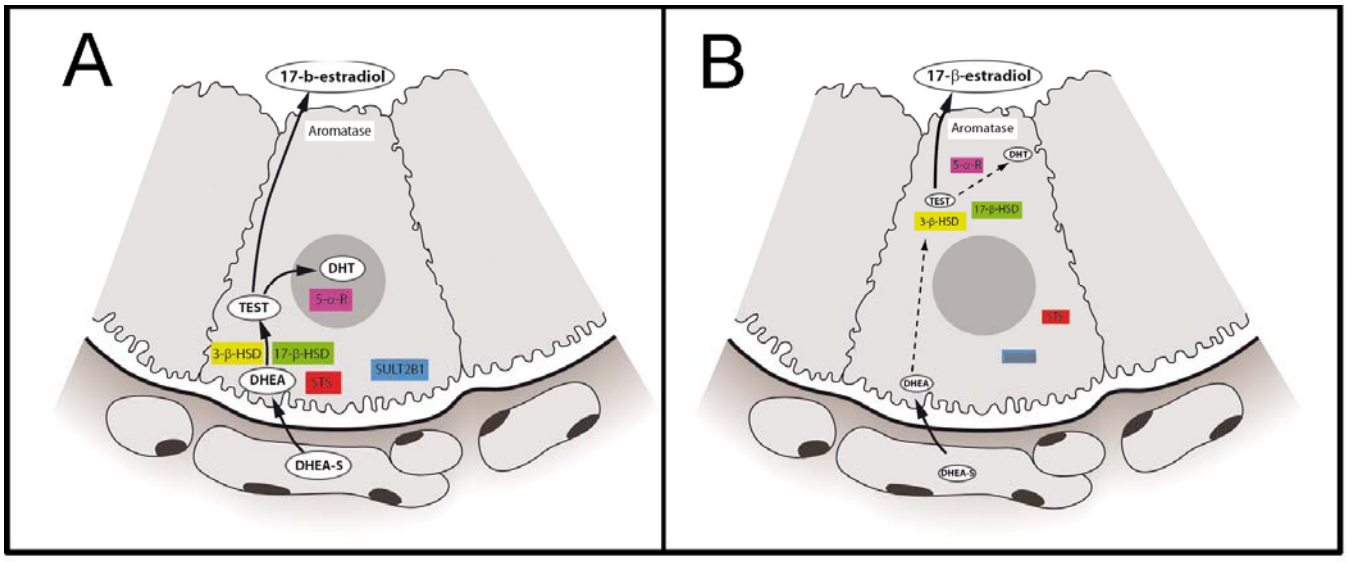

Figure 7. Local intracrine enzymatic machinery in salivary gland acinar cells in healthy individuals and in patients with Sjögren's syndrome (SS). Panel A: In healthy acinar cells intracrine enzymes are organized logically with enzymes acting in the beginning of DHEA processing localized in basal parts of the cells and dihydrotestosterone synthesizing 5-α-reductase situated in nucleus. Panel B: In SS acinar cells expression of androgen processing intracrine enzymes is lower and enzymes have lost their architecture. Routes marked with a dashed line are impaired leading to lower androgen concentrations. Localization of 17-β-estradiol producing aromatase is unchanged. DHEA-S = dehydroepiandrosterone sulfate, DHEA = dehydroepiandrosterone, Test = testosterone, DHT = dihydrotestosterone, STS = steroid sulfatase, SULT2B1 = sulfotransferase 2B1, HSD = hydroxy steroid dehydrogenase, 5-α-R = 5-α-reductase.

At the mRNA level the differences between SS patients and healthy controls were not as striking. We used RNA from whole labial salivary glands so we were not able to examine the expression patterns specifically in acinar or ductal cells. However, in SS labial salivary glands the expression of aromatase mRNA was decreased compared to healthy controls and consequently the expression of 5-α-reductase mRNA in SS glands was higher than aromatase. Despite that, immunohistochemical staining of aromatase did not show any differencies in acinar cells between the healthy and diseased glands. In addition to aromatase, the expression of 3-β-HSD mRNA was decreased in SS.

Since the expression and localization of the intracrine enzymes in SS labial salivary glands were shown to be deranged, we hypothesized that this defect would also be seen in the function of this intracrine machinery. We confirmed our hypothesis and showed that in SS the correlations between salivary DHEA and testosterone and salivary testosterone and DHT in SS, supposed to reflect the situation in salivary glands, were not similar to the healthy glands. In SS glands salivary DHEA levels correlated with salivary testosterone levels, which was not seen in healthy glands, and at the same time the correlation between salivary testosterone and DHT was lower than in healthy glands. The same phenomenon was evident when we examined DHEA/testosterone and testosterone/DHT ratios. The former was lower in SS patients showing more effective conversion of DHEA to testosterone in SS salivary glands than in normal glands. On the contrary, in SS patients the testosterone/DHT ratio was higher compared to the healthy individuals suggesting faulty local conversion of testosterone to DHT by 5-α-reductase.

Deranged function of the intracrine machinery in SS salivary glands *in vivo* was seen also as an inability to locally convert oral DHEA to active androgens. Previously, oral DHEA replacement therapy (50 or 100 mg *q.d.*) has been shown to lead to increased serum concentrations of androgens, but not estrogens, in healthy women. On the contrary, in men the concentrations of both androgens and estrogens remained fairly unchanged after DHEA treatment (Morales *et al.*, 1994; Morales *et al.*, 1998). Additionally, percutaneous DHEA therapy has been shown to have an androgenic but not an estrogenic action in healthy elderly women. Moreover, DHEA has been shown to be converted mainly to conjugated metabolites such as androsterone-glucuronide (ADT-G) and androstane 3α,17β-diol- glucuronide (3α-diol-G), which are considered to reflect the intracrine production of androgens better than active androgens (Labrie *et al.*, 1997b; Labrie *et al.*, 2007). We showed that the DHEA treatment led to normal or to slightly increased serum concentrations of all androgens and their metabolite, but the effect on local (salivary) levels of androgens was minor. This absence of correlation between serum and salivary androgens in SS is contrary to healthy women, in whom the salivary concentrations reflect the systemic concentrations (Swinkels *et al.*, 1988; Rilling *et al.*, 1996; Granger *et al.*, 1999; Lewis, 2006; Ahn *et al.*, 2007).

In SS patients serum concentrations of DHT reached the normal values and those of DHEA-S, androstenedione and 3α-diol-G even exceeded normal concentrations after the DHEA

treatment. Concentrations of free testosterone remained under the reference values even after the DHEA replacement therapy (figure 8).

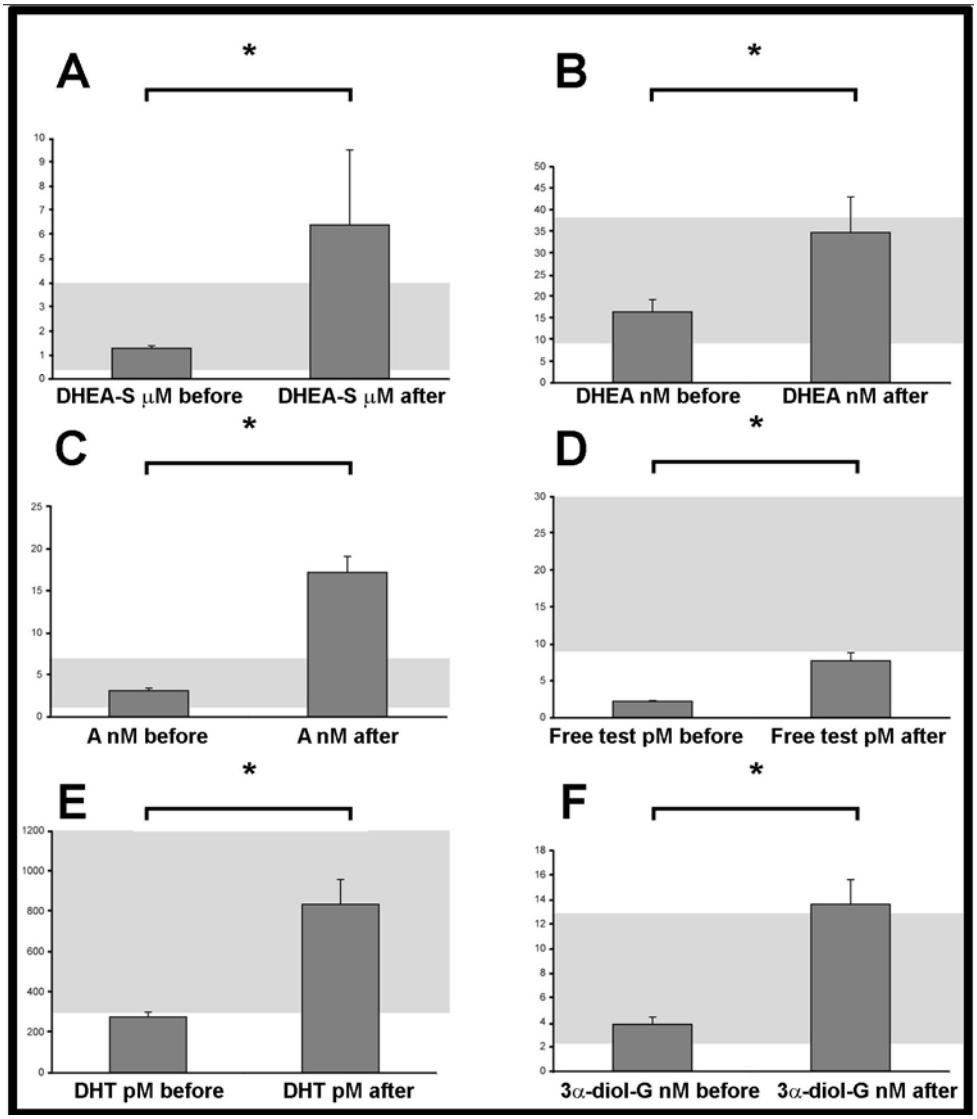

Figure 8. Effect of a 4-month replacement therapy with dehydroepiandrosterone (DHEA) (50 mg / day) on serum levels of DHEA-sulfate (A), DHEA (B), androstenedione (C), free testosterone (D), dihydrotestosterone (E) and androstane 3α,17β-diol-glucuronide (F) in patients with Sjögren's syndrome. Reference values are shown in grey background. * = statistically significant. A = androstenedione, test = testosterone, DHT = dihydrotestosterone, 3α-diol-G = androstane 3α,17β-diol-glucuronide.

However, salivary outputs (amount of secreted hormone in 5 minutes) of only DHEA and DHT increased significantly (from 5.45 ± 0.82 to 11.14 ± 3.02 pmol, p=0.028 and from 0.91 ±

0.20 to 1.44 ± 0.34 pmol, p=0.028, respectively) but the change was clearly minor compared to changes in the serum values. Importantly, in some individuals (25%) no increases in local androgens were seen at all, besides the increase of DHEA itself (figure 9), although the same patients showed increases in serum levels of androgens. Thus, there seem to be differences between the individual patients in local androgen processing capacity and in some patients this capacity seems to be almost unsubstantial.

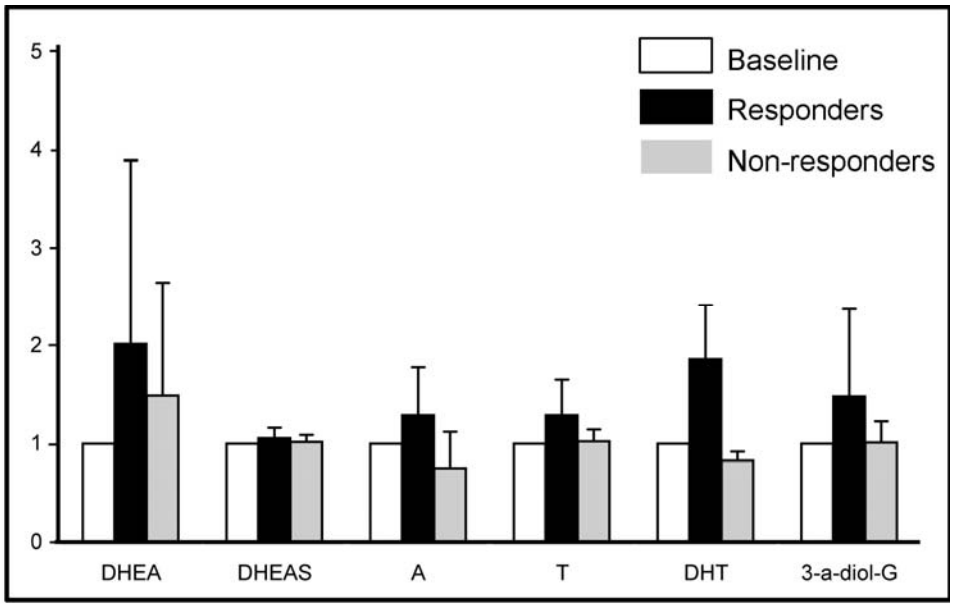

Figure 9. Relative changes in salivary levels of androgens after dehydroepiandrosterone (DHEA) replacement therapy in patients with Sjögren's syndrome responding (black columns, n=9) and not responding (grey columns, n=3) to DHEA therapy. White columns represent baseline levels and are given a value of 1. Black and grey columns represent salivary androgen levels after the DHEA treatment and are in proportion to baseline values. DHEA-S = dehydroepiandrosterone sulfate, A = androstenedione, Test = testosterone, DHT = dihydrotestosterone, 3-a-diol-G = androstane 3α,17β-diol-glucuronide.

Our results suggest that the 5-α-reductase defect has a strong effect on the local androgen deficiency seen in SS salivary glands. Deficiency of type II 5-α-reductase in humans leads to pseudohermaphroditism in males whereas females seem endocrinologically normal (Imperato-McGinley and Zhu, 2002). However, as mentioned above, we showed that 5-α-reductase in salivary glands is of type I, the deficiency of which has not been described in humans.

7.2.3. Eventual causes of the intracrine defect in Sjögren's syndrome

One potential disturbing factor in the SS salivary gland intracrinology is the increased presence of cytokines, which can disturb the DHEA processing enzymatic machinery as mentioned earlier. On peripheral level cytokines are generally considered to increase the activity of aromatase and consequently shift the production of active sex steroids towards estrogens, as implied also in our study. Cytokines that have been reported to increase the activity of peripheral aromatase include TNF, IL-1 and IL-6, among others (Macdiarmid *et al*., 1994; Purohit *et al*., 1995). The increased activity of peripheral aromatase can lead to lower androgen and higher estrogen concentrations in the target tissue of SS. Besides aromatase, cytokines affect other steroidogenic enzymes as well. The function of 17-β-HSD has been shown to be stimulated by EGF, IFN-γ, TGF-α and TGF-β and that of 5-α-reductase by TGF-β1 ja TGF-β2 (Herrmann *et al*., 2002). Additionally, TNF-α has been shown to inhibit the conversion of DHEA-S to DHEA in RA synovial cells and thus cause decreased local concentrations of DHEA and further decreased production of active sex steroids from this prohormone (Weidler *et al*., 2005). Cytokines are also considered to direct the metabolism of estrogens into 16α-hydroxylated forms, which are proinflammatory (Janele *et al*., 2006).

Besides the function of the DHEA processing machinery, the uptake of steroids into SS salivary glands can be affected. The most common route of entry for neutral sex steroids to saliva has been considered to be diffusion through the plasma membranes and the acinar cells. As mentioned earlier, megalin mediated uptake represents another mechanism for the entry of SHBG-bound sex steroids into salivary glands (Hammes *et al*., 2005). Taking into account that the vast majority (98 -99.5 %) of serum sex steroids are bound to SHBG (Dunn *et al*., 1981), megalin-mediated transport could be of major relevance for the salivary gland sex steroid environment and further for the physiology of these glands.

Uptake of DHEA seems to be normal in SS as shown by elevated output of DHEA after the replacement therapy in all SS patients studied. Since the DHEA replacement therapy significantly increased the outputs of only DHEA and DHT in SS patients, the existence of DHEA and/or DHT binding receptor in salivary glands is possible. Defects in the entry of active steroids such as DHT into salivary glands could further ameliorate the local androgen depletion caused by the defective local androgen production. This could be the situation in the

subpopulation of patients in whom no increases in salivary hormones besides DHEA was seen after the DHEA treatment. Active transport is possible also for polarised DHEA-S, which is unable to diffuse from circulation to salivary gland cells (Lewis, 2006). Organic anion transporting polypeptides such as OATP-2B1 have been suggested to be involved in the uptake of DHEA-S into saliva (Pomari *et al.*, 2009). If existing, this active transport could be somehow disturbed in SS salivary glands (Figure 10). All together, the expression and function of active sex steroid transporters in SS should be studied.

Besides the above-mentioned defective local processing of DHEA and the decreased uptake of steroids, low increase in salivary output of androgens might have been due to accelerated metabolism of androgens into their conjugates by phase II metabolizing enzymes. However, we showed that this is not true in SS salivary glands, shown by the incapability of the DHEA treatment to significantly increase the local output of metabolite 3α-diol-G, although its systemic concentrations were increased. This demonstrates that increased conjugation of androgens is not causing the local androgen depletion in SS salivary glands.

7.2.4. Intracrine defect and the female dominance of Sjögren's syndrome

Intracrine defect can be detrimental especially for women. As mentioned earlier, the local intracrine production of active sex steroids becomes increasingly important in older people, especially in postmenopausal women (Belanger *et al*., 1986; Labrie, 1991). These women are deprived of both systemic estrogens and androgens due to the declining function of the ovaries and the adrenal gland, respectively. Furthermore, women are more dependent on local sex steroid production than men who have testis derived testosterone produced throughout their lives. Testosterone is only one enzymatic reaction away from the androgenic and estrogenic end products, DHT and 17-β-estradiol, respectively. Women, instead, have first to produce DHEA and then convert it to active sex steroids through many intermediates and are thus more prone to defects in this local processing. As the conversion of testosterone to DHT seems to be defective in SS, testis derived testosterone provides men with more substrate and thus possibly protects them against the DHT depletion caused by this SS-related intracrine failure. Since most patients with SS are middle-aged women, disturbances in peripheral sex steroid production have a significant effect on their local sex steroid levels.

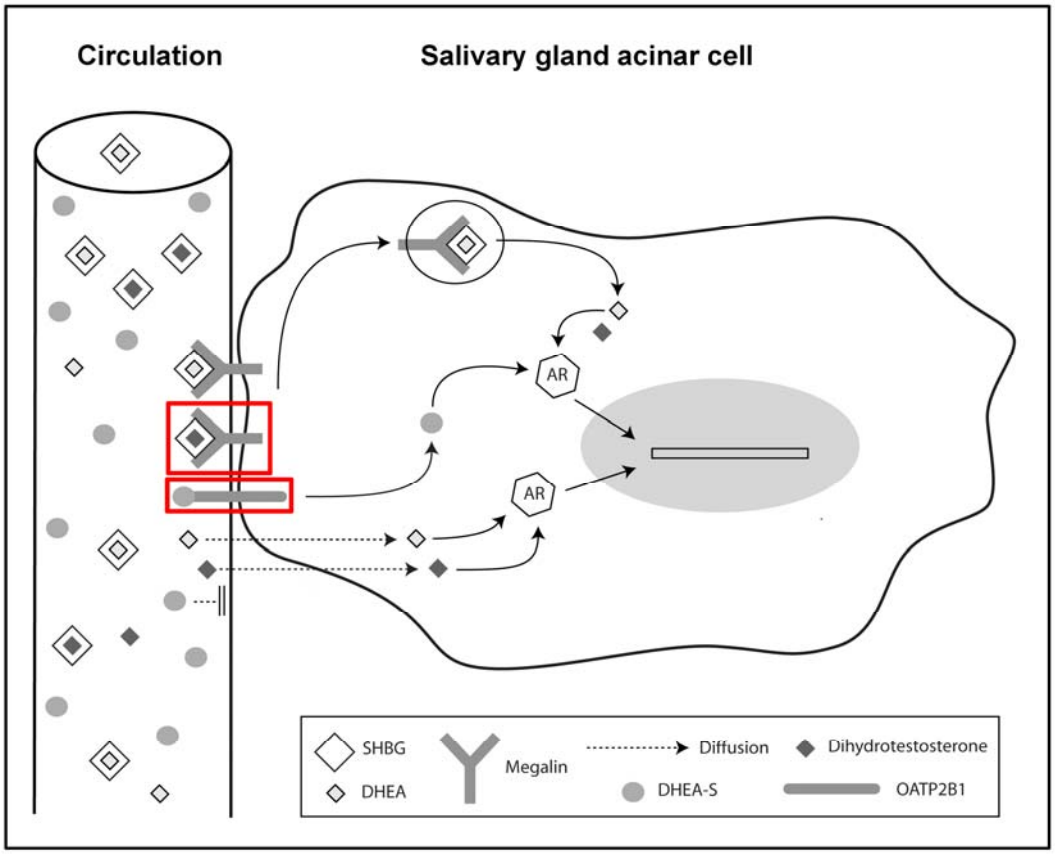

Figure 10. Schematic picture of the uptake of androgens in salivary gland acinar cell. Neutral steroids such as dehydroepiandrosterone (DHEA) and dihydrotestosterone diffuse to epithelial cells whereas polar steroid such as DHEA-sulfate (DHEA-S) are uncapable of diffusion. Megalin may be responsible for the possible active uptake of sex-hormone bingding globulin (SHBG) –bound steroids into salivary gland cells and OATP2B1 for the active entry of DHEA-S. In a subgroup of patients with Sjögren's syndrome, characterized with no response in local androgen levels to oral DHEA –therapy, megalin- and/or OATP2B1-mediated active uptake of active androgens and DHEA-S, respectively, might be affected (shown in red). On the contrary, the uptake of DHEA seems normal. After entering the cell cytoplasm, androges bind to androgen receptor (AR) and regulate the transcription of their target genes. SHBG = sex-hormone bingding globulin, DHEA = dehydroepiandrosterone, DHEA-S = dehydroepiandrosterone sulfate, OATP2B1 = organic anion transporting polypeptide 2B1, AR = androgen receptor.

7.3. Effect of androgens on Sjögren's syndrome related characteristics *in vivo* (I, III-IV)

Androgen deprivation is likely to have various effects on patients with SS. We studied the associations between androgens and SS related characteristics and the effects of the oral DHEA replacement therapy on these characteristics in SS patients.

7.3.1. Androgen levels reformulate salivation but not autoantibody production in Sjögren's syndrome patients

Many of the earlier studies report decreased salivary flow rates in menopausal women but an increase in these rates during HRT, suggesting an influence of estrogens on salivation (Eliasson *et al*., 2003; Yalçin *et al*., 2005). Also a negative correlation between serum estrogen levels and oral symptoms in SS has been reported (Tayim *et al.,* 2004) and some studies report no effect of menopause or HRT on salivation (Ship *et al*., 1991; Eviö *et al*., 2006). Association between serum or salivary androgen levels and salivation has been less studied. Both lowered salivary levels of DHEA and salivary flow rates have been shown in patients with the burning mouth symptom but these two were not correlated either in patients or in healthy controls (Dias Fernandes *et al*., 2009). In a recent study a negative correlation between serum testosterone levels and symptoms of dry mouth was shown (Forsblad-d'Elia *et al*., 2009). In the same study, symptoms of dry mouth were shown to relieve after DHEA replacement therapy, as has been shown earlier (Pillemer *et al*., 2004).

In our study, salivary outputs of testosterone, DHT and 3α-diol-G proved to correlate with both resting and stimulated salivation and the treatment with DHEA did not affect this correlation. Local salivary levels of testosterone and DHT were normalized upon DHEA therapy, which emphasizes also the role of a functional intracrine machinery processing DHEA further to testosterone and DHT in the function of salivary glands.

Serum levels of androgens and estrogens have been demonstrated to correlate inversely with SS-autoantibodies (Forsblad-d'Elia *et al*., 2009) so we studied the effect of the 4-month long DHEA replacement therapy on them in SS patients. However, in our study in most patients no

autoantibody response to DHEA was observed, although in 2 out of 12 patients studied there was a slight decrease in the titers of SS-A or SS-B autoantibodies.

7.3.2. Dehydroepiandrosterone treatment is not superior to placebo in the treatment of fatigue in Sjögren's syndrome patients

One of the most disturbing and restrictive symptoms in SS is severe fatigue, the reason of which is still unknown. Possible causes for SS-related fatigue include defects in the autonomic nervous system (Barendregt *et al*., 1998), cytokines (Baturone *et al*., 2009) and psychological factors such as depression (Barendregt *et al*., 1998). One potential cause of fatigue in androgen deficient SS could be hormonal imbalance. Weak androgens such as DHEA have been reported to improve fatigue and wellbeing in the elderly (Morales *et al*., 1994) and in Addison's disease (Arlt *et al*., 1999; Hunt *et al.,* 2000). Moreover, serum levels of DHEA-S, but not testosterone, have been shown to positively correlate with the quality of sexual life and mental wellbeing in women with pSS (Valtysdóttir *et al*., 2003). We hypothesized that DHEA replacement therapy could improve fatigue in DHEA-deficient SS patients.

DHEA proved not to be superior to placebo in the treatment of fatigue or in improving the health-related quality of life in SS patients with severe fatigue and low DHEA-S concentrations. Congruently, no correlation between systemic or local androgen levels and MFI scores in SS patients was seen. Our results are congruent with previous results (Pillemer *et al*., 2004; Hartkamp *et al*., 2008), which reported DHEA therapy to be non-beneficial in the treatment of fatigue of SS patients. However, the study setting in these studies was completely different from our study, in which SS patients had both severe fatigue and subnormal DHEA concentrations, and a lower substitution dose of DHEA was used.

These negative results do not exclude the possibility of the contribution of androgen depletion to SS-related fatigue. Absence of correlation between androgen levels and MFI scores might derive from the great interindividual variation in hormonal levels. Also the aforementioned fact that in a proportion of SS patients no signs of local intracrine activity was observed could contribute to lack of correlation. However, our results do demonstrate the incapability of oral DHEA replacement therapy to improve fatigue experienced by patients with SS. This incapability could be explained by defective local processing of DHEA into active sex

steroids in peripheral tissues such as salivary glands in SS patients as stated above. If this defect is seen also in other peripheral tissues such as brain, it is logical that the restoration of the systemic DHEA levels does not lead to restoration of local concentrations of DHT or other active androgens in patients with SS. However, one valuable conclusion of our study and another study (Hartkamp *et al*., 2008) is the ability of the placebo treatment to ameliorate fatigue and health-related quality of life in pSS patients. This event shows that treatment by a specialist and the feeling of a potential to influence the course of their disease can help SS patients in controlling fatigue and depression.

7.4. Androgen regulation of laminin-111 and integrins α1β1 and α2β1 in salivary glands (V)

Since patients with SS have local androgen depletion in salivary glands, we wanted to study the effect of this hormonal imbalance in the target tissue of SS. As mentioned earlier, salivary glands are regulated by androgens (Treister *et al*., 2005) and acinar and ductal cells seem to be potential target of androgens in salivary glands (Laine *et al*., 1993). Accordingly, we assumed that androgens are important to the maintenance and function of salivary gland acinar cells. Specifically, we hypothesized that androgens affect the interaction between the acinar cells and BM through integrins α1β1 and α2β1 and laminin-111, the expression of which we have earlier shown to be decreased in SS (Laine *et al*., 2004; Laine *et al*., 2008).

7.4.1. Sjögren's syndrome affects salivary glands

As mentioned earlier, in SS salivary glands are strongly affected. In addition to immune infiltrates, central characteristics of SS salivary glands include structural changes, acinar atrophy and concordant ductal cell hyperplasia contributing to the diminished secretory function.

Accelerated apoptosis of the acinar cells in SS salivary glands has been suggested and could lead to redistribution and further to presentation of autoantigens and breakdown of immunological tolerance (Ohlsson *et al*., 2002). However, also studies showing that apoptosis of the epithelial cells in SS glands is rare are available, possibly at least partly due to

heterogenic patient material and varying methods used in the assesment of apoptosis (Ohlsson *et al*., 2001).

Another potential contributor to acinar cell loss in SS is defective remodelling of SS salivary glands. Normally salivary glands remodel continuously and the lost acinar cells are thought to be replaced by the migration and differentiation of the relatively undifferentiated intercalated ductal epithelial cells (Man *et al*., 2001). Signals for this acinar transdifferentiation are believed to come locally from the underlying BM. In SS, however, this differentiation process is believed to be defective. The undifferentiated ductal cells are suggested to divide and replace the acinar cells with cells, which due to their inability to differentiate to acinar cells retain the intercalated duct cell phenotype (Manganelli and Fietta, 2003). Finally, the outcome is acinar cell atrophy and loss and a simultaneous ductal cell hyperplasia (Polihronis *et al*., 1998; Man *et al*., 2001).

Also BM is strongly affected in SS. In the diseased glands disorganisation or patchwise even total absence of the BM has been reported, especially in patients with low interacinar fibrosis reflecting early stages of the disease (Kwon *et al*., 2006). BM around ducts and acini has been reported to be thinner and glandular structures detached from the basal lamina (McArthur *et al*., 1997; Goicovich *et al*., 2003). In addition, changes in the acinar specific ECM laminins and their cellular integrin receptors (laminin-111 and integrins α1β1 and α2β1, respectively) have been reported (Laine *et al*., 2004; Laine *et al*., 2008; Velozo *et al*., 2009). The expression of BM modifying MMP-3 and MMP-9 has been reported to be increased in SS glands (Konttinen *et al*., 1998; Perez *et al*., 2000), which may lead to increased degradation of the BM, disturbances in the polarization of the tubuloacinar epithelial cells and to dysregulated signaling between the glandular cells and ECM (Garcia-Carrasco *et al*., 2006).

We hypothesized that androgen depletion could contribute to these pathogenic BM alterations in SS salivary glands. Thus, we studied the role of androgens in the structural abnormalities involving the acinar BM specific ECM molecules and their cellular receptors (laminin-111 and integrins α1β1 and α2β1, respectively).

7.4.2. Androgens upregulate integrin subunits α1 and α2

We demonstrated that in salivary gland cells and in healthy labial salivary glands androgens, but not estrogens, upregulate integrin subunits α1 and α2. These subunits in heterodimers with the β1 subunit are central for the communication of acinar cells with its BM through their interaction with acinar cell specific laminin-111 (Hoffman *et al*., 1996). The expression of both acinar BM laminin-111 and acinar cell integrins α1β1 and α2 β1 has been earlier shown to be diminished in SS salivary glands (Laine *et al*., 2004; Laine *et al*., 2008). We thus demonstrated a connection between two characteristic features of SS, androgen depletion and defects in molecules enabling the signaling between the acinar cells and the acinar BM (Figure 11).

The most potent androgenic upregulator of the integrins studied in ductal and acinar cells was DHEA, followed by testosterone. The most active androgen, DHT, was not as effective. This could be due to non-optimal dose or time points used. Alternatively, the entry of DHT into cells by passive diffusion or its active transport could affect the results as could the intracrine balance between androgens and estrogens. When the effect of DHEA was studied on tissue level, upregulation of integrin subunits α1 and α2 by DHEA was seen in healthy labial salivary glands but not in glands from the SS patients, suggesting faulty local intracrine DHEA processing in SS as also suggested by our earlier studies.

In addition to androgens, also the contact between the cells and BM extract Matrigel, which contains laminin-111, increased the expression of integrin α1 and α2 subunits. This suggests that both the contact with laminin α1 chain and the presence of DHEA and its metabolites are needed for proper cell-BM-communication. Also the expression of laminin α1 chain by epithelial cells was increased by contact with Matrigel, indicative of a self-amplifying positive feedback mechanism in laminin α1 chain/-111 expression. This system could enable the maintenance of laminin-111 in acinar BMs, which further site-specifically guides the progenitor-to-acinar cell differentiation. In contrast to integrins, expression of laminin-111 was not affected by androgens.

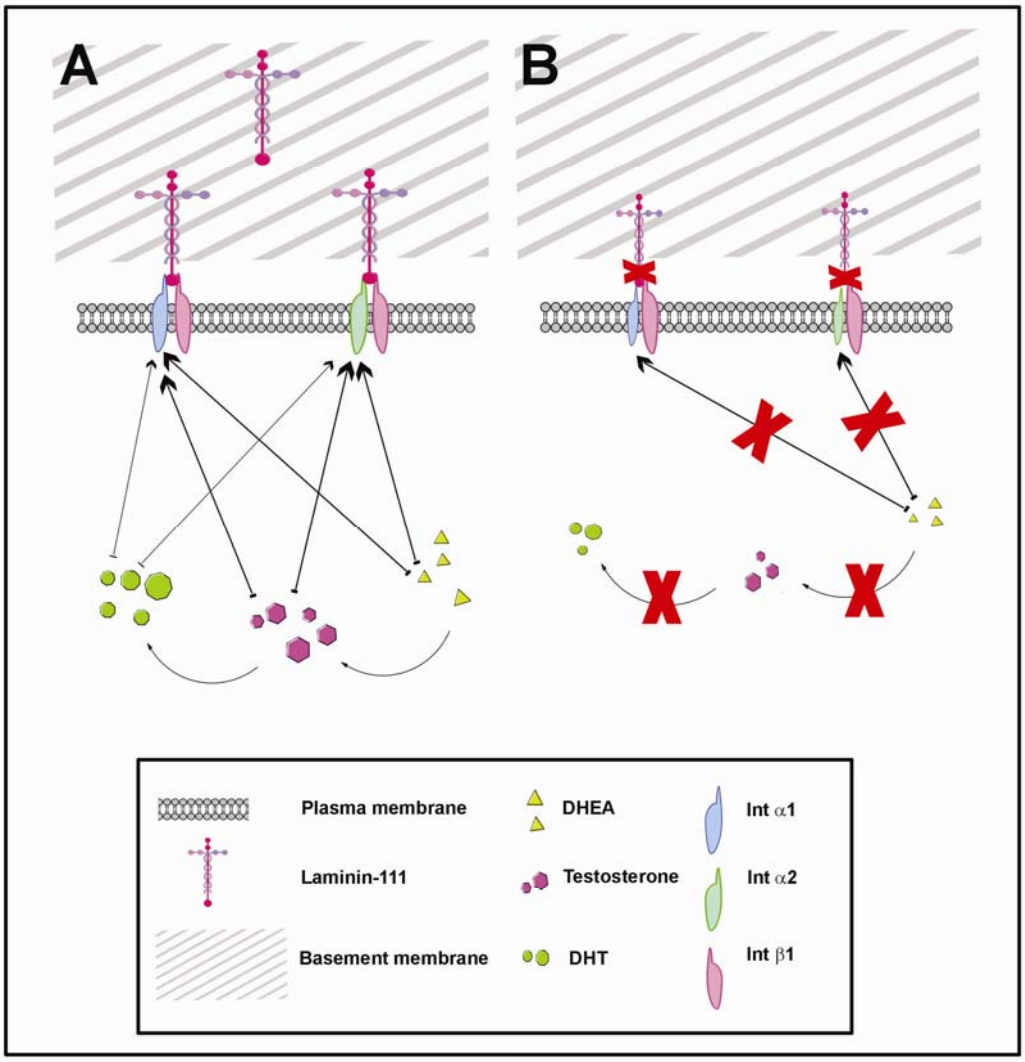

Figure 11. Schematic picture representing the connection between laminin-111 and integrin α1β1/α2β1 and the effect of androgens on integrin expression in salivary gland acinar cells. In acinar cells from healthy salivary glands (panel A) there is local synthesis of active androgens (testosterone and dihydrotestosterone (DHT)) from the adrenal gland derived dehydroepiandrosterone (DHEA). In healthy salivary glands androgens, especially DHEA and testosterone, upregulate integrin subunits α1 and α2 (shown in the picture with arrows with a thick arrowheads). Conversely, in acinar cells from patient with Sjögren's syndrome (panel B) the expression of the acinar specific integrins α1β1 and α2β1 and laminin-111 is decreased impairing the communication between the acinar cells and their basement memrane. Additionally, androgen depletion, defective local androgen processing and faulty androgen regulation of the integrin subunits further deteriorates the situation. Int = integrin.

Androgen depletion and defective androgen processing in SS salivary glands, combined with the lack of laminin-111 and integrins α1β1 and α2β1, impair the signaling between the BM and acinar cells. This defect might lead to impaired maintenance of the acinar cells and further contribute to the acinar cell atrophy and ductal epithelial cell hyperplasia seen in SS salivary glands. Androgen depletion may be the reason behind the deficient acinar maintenance caused by disturbances in salivary gland BM and its cellular receptors, which have already been reported before (Laine *et al*., 2004; Laine *et al*., 2008).

Androgen depletion and defects in the integrin signaling molecules in SS might also account for the hypofunction of the SS salivary glands through a different route than by impairing acinar maintenance. Laminin-111 participates in the stabilization of synapses (Sugiymama *et al*., 1997; Lee *et al*., 2002). Thus laminin-111 depletion in SS (Laine *et al*., 2004), combined with defects in its cellular integrin receptors (Laine *et al*., 2008) could impair the signaling between the parasympathetic nerve terminals and postsynaptic muscarinic receptor and thus affect salivation in SS salivary glands.

8. SUMMARY AND CONCLUSIONS

Etiopathogenesis of SS has remained obscure in spite of extensive research.We hypothesized that sex steroids play an important role in the etiopathology of SS and affect the state of the diseased salivary glands. Thesis study confirmed our hypothesis and at least to some degree clarified the enigma of SS and the status of SS salivary glands by showing that patients with SS are androgen depleted. This androgen defect is seen on a systemic level and, even more importantly, on a tissue level in the target tissue of SS, salivary glands. Thus, SS patients have both a defective endocrine arm and an impaired intracrine arm due to more severe adrenopause and defective intracrine machinery, respectively. In contrast to androgen levels, systemic and local concentrations of estrogens in patients with SS are slightly higher than in healthy individuals thus lowering the androgens-to-estrogen ratio. The importance of the local androgen environment to salivary glands is clearly seen in SS, in which salivary enzymes participating in androgen production are affected. On the contrary, the expression of 17-β-estradiol producing aromatase seems to be relatively unaffected. The hormonal imbalance caused by androgen deprivation combined with a relative estrogen surplus might predispose SS patients to autoimmune aggression.

We believe that especially the local intracrine defect in DHEA processing is central for the pathology of SS. We have shown that in SS, unlike in healthy individuals, the effect of systemic sex steroid levels on local levels in salivary glands is not straightforward. Therefore, our results suggest that the implementation of oral DHEA replacement therapy in the treatment of SS is unsubstantial as to the treatment of fatigue and restoration of the local sex steroid balance, most probably due to the intracrine failure in local androgen production.

The pathogenetic model of SS should be able to explain the structural changes and the diminished function of the affected glands. In this thesis we demonstrated a connection between androgen depletion and defects in SS salivary glands. We showed that androgens contribute to the expression of acinar cell signaling molecules, integrin subunits α1 and α2, through which the acinar cells communicate with the underlying BM. Additionally, we showed that local androgen levels, especially outputs of the two most active androgens, testosterone and DHT, correlate positively with salivation. Thus, local conversion of DHEA further into active androgens has an impact on salivary gland structure and function and defects in it can at least partly explain the diminished salivary secretion in SS.

Androgen depletion and especially local intracrine defects in SS could also explain the female dominance of the disease. Since the local conversion of testosterone to DHT seems strongly affected in SS, the testosterone excess in men compared with women provides them with more substrate and thus protects men from the eventual endocrine and intracrine defect. Women, on the other hand, are more dependent on local DHEA processing and are thus predisposed to defects exposing to SS.

Signs of the etiopathology of SS might not be unambiguous. SS patients seem to differ in the amount of the intracrine defect in salivary glands: some patients did not have any processing of DHEA further to active androgens, referring to more severe local androgen defect, whereas in the majority of patients some intracrine activity was seen.

Finally, primary and secondary forms of SS presumably differ in their etiologies. We believe that the local intracrine defect affects the exocrine glands of pSS patients. When the systemic concentrations of androgens remain sufficiently high, glands are fed with enough pro-hormones and active androgens and acinar maintenance and function are ensured. However, when menopause and a concomitant early and/or more severe adrenopause take place, glandular welfare is shaken, pathological processes are induced and symptoms appear. sSS, on the contrary, could derive from the inflammation-related reduction in the adrenal prohormone synthesis. Long-lasting inflammation and associated cytokines can damage the adrenal cortex and decrease the amounts of DHEA(-S) and androstenedione secreted. A systemic androgen deficiency would follow leading also to local androgen depletion in salivary and other exocrine glands, predisposing the affected glands to autoimmune attack. We thus believe that pSS is caused by a local intracrine androgen deficiency, the level of which varies between individuals, whereas sSS might represent a secondary deficiency originating from a systemic androgen defect.

9. ACKNOWLEDGEMENTS

I am most grateful to my supervisor Professor Yrjö T. Konttinen who has been the origin and support of my studies. Besides being the most intelligent person that I know, you also have a big heart, a great sense of humour and a strong confidence in us. You have created a unique atmosphere in the lab so that it has been nice to come to work every day! I also want to thank my other supervisor, Docent Dan C.E. Nordström for the support and guidance during these studies. Thank you for always being ready to help me when I have needed it. Late Professor Ismo Virtanen is acknowledged for his invaluable help in the laboratory, especially with the cell work.

Professor Anne Isine Bolstad is warmly thanked for approving the invitation to be my opponent and travelling to Helsinki from Bergen, Norway. I am honoured to have a Sjögren's specialist discussing with me and I hope we will have a nice time in Helsinki.

Professor Juha Tapanainen from Oulu and Professor Roland Jonsson from Bergen, Norway, are deeply thanked for the revision of my thesis. Thank you for sharing your wide knowledge and experience with me and for guiding me in the writing process.

My deep thanks go to Mari. Thank you for teaching me from the beginning how to work in laboratory and also how to have fun while pipetting. I admire your patience and kindness. I am grateful to Jami for teaching me how to think scientifically and giving me valuable advices on life in general. I want to thank Eija for help, friendship and great company both in and outside of lab. We share a common sense of humour and a similar attitude towards life and your right words at the right time have saved my day many times. Liisa is acknowledged for having patience to help me with my favourite subject, statistics, time after time. I am most grateful to Erkki and Emilia for understandingly assisting me with my other favourite, Photoshop, and Mikko and Mika for helping me with the microscope. Mikael and Vasily are acknowledged for good collaboration in the field of Sjögren's syndrome and Hanna-Mari for assistance in many practicalities. I am thankful to Teppo and Jukka for aiding me and my dearest, sometimes from the other side of Finland. I also want to thank other members, past and present, of the TULES group: Eero, Guofeng, Kalle, Katja, Noe, Praseet, Raimo, Tarvo, Vesa-Petteri, Yuya and everybody else. I honestly think that I have the best workmates there

can be. You have all made these years special for me and I feel privileged to be able to work with you. Together we have made every day, and some evenings, spent together immemorial!

My great acknowledgements go also to my co-workers both in Finland and abroad: Masayuki Azuma, Helena Forsblad-d'Elia, Göran Kvist, Tucker Patterson, Antti Pihakari, Beata Przybyla, Blaz Rozman, Svetlana Solovieva, Michelle Spaan, Rainer Straub, Sigridur Valtysdottir and Eva Waltbrand. I am also thankful to the people in the Department of Anatomy, especially Outi, Pipsa, Minna and Reijo for helping me in my studies.

I want to say special words of thanks to my father Erkki, who has always believed in me and has never questioned me in anything I have done. Thank you also for always being there for me whenever I have needed it. I also want to thank my mother Pirjo in heaven. You would have been very proud of me now and I know that you are with me everywhere I go.
I also owe warm thanks to Seija for making me feel beloved and Seppo, Tuula and Otto for welcoming me and Mooses to their family.

To all my “off-lab” friends Pia-Marjut, Jenni, Sussu, Maija, Janica, Satu, Veera, Hanna, Pauliina, Päivi, Heini, Tintti, Markus, Suvi, Viki, Nadja, Niina, Heidi, Tapsa, Laura, Jenni, Risto, Tiina, Teemu, Tiina, Janne, Otto, Eeva, Timo, Piia, Matti, Pete and everybody else: you are important to me. You have made sure that I have not concentrated only to pipetting but have lived life to the full in every way and every day (and night). I want to thank you for being with me in better and worse during all these years. I especially want to thank Susanna and Janica for our days in Biomedicum and other work-related environments both in- and offshore. Päivi, Tintti, Heini, Markus, Veera and Hanna are thanked for making my studying years in Viikki easier and, in particular, more enjoyable. I am very grateful to Matti for the help with the figures in the thesis. I also want to give special thanks to my little sweetheart Unna.

Last but not least, I want to thank Mikko. I love you and I thank you for always being there for me and laughing and crying with me. Together we’re invincible.

This study was supported by Biomedicum Helsinki Foundation, Emil Aaltonen Foundation, Finnish Cultural Foundation, Finska Läkaresällskapet, Helsinki University Central Hospital (EVO funding), Maire Lisko Foundation, National Graduate School of Musculoskeletal Disorders and Biomaterials, Orion-Farmos Research Foundation and University of Helsinki.
I am also very thankful for the support I have got from the Finnish Society for Rheumatology.

Helsinki 17.2.2011

Pauliina

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